ATP AND ITS RECEPTORS IN NERVE INJURY AND REPAIR

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ABSTRACT

Unlike the peripheral nervous system (PNS), adult neurons in the central nervous system (CNS) have limited regenerative capacity after injury. One interesting phenomenon observed nearly four decades ago was that lesion of a peripheral nerve can significantly enhance the regenerative capacity of the central axons of the corresponding dorsal root ganglion (DRG) neurons, termed a 'conditioning lesion', but the underlying mechanism is still not fully understood. Since ATP is released after nerve injury and extracellular ATP has a broad range of biological activities, we postulated that ATP might be the injury signalling molecule that triggers the regenerative machinery in the injured neurons. If that were the case, injection of ATP into a peripheral nerve should be able to mimic the effect of a conditioning lesion. To test this theory, we injected ATP into a peripheral (sciatic) nerve after a dorsal column transection and found that ATP injection did promote the regeneration of injured axons into the lesion cavity. We also found that ATP injection activated transcription factor STAT3 and increased the expression of growth associated protein 43 (GAP43) in the corresponding DRG neurons. ATP injection increased the concentrations of ciliary neurotrophic factor and interleukin-6 in sciatic nerve and DRG. These results indicate that intraneural injection of ATP can mimic conditioning lesion to a certain degree. Most interestingly, we found that a second injection of ATP one week after the first one markedly boosted the effects of the first injection as many more axons grew into or across the lesion compared with double saline injection or ATP plus saline injection. Double ATP injection is also more effective in sustaining the expression of phospho-STAT3 and GAP43. Immunohistochemical analysis showed ATP injection caused little Wallerian degeneration at the injection site. Behavioural tests showed no long-term adverse effects to the injected sciatic nerve. In order to explore the underlying mechanism of ATP

induced elevation of the regeneration state of DRG neurons and look for more potent purinoceptor agonists to stimulate axonal regeneration, we first tried to identify the expression of purinoceptor subtypes in sciatic nerves using quantitative PCR and immunohistochemistry. We found that mRNAs for all the four P1 and fourteen P2 purinoceptor subtypes were expressed in the sciatic nerve, DRG or dissociated Schwann cells at various levels. Immunohistochemical analysis showed that purinoceptor subtypes are expressed by different types of cells. Due to the expression of nearly all purinoceptor subtypes in the sciatic nerve, it will be a big challenge to identify the receptor subtype(s) responsible for ATP induced axonal regeneration. We have set up a compartmented co-culture system to test various agonists/antagonists of purinoceptors. Taken together, we have shown that intraneural ATP injection can mimic conditioning lesion in promoting sensory axonal regeneration. Identification of the receptor subtype(s) and other molecules involved in the enhanced regeneration capacity of injured neurons may lead to the development of therapeutic agents to effectively promote the axonal regeneration of both peripheral and central neurons.

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LIST OF ABBREVIATIONS

DABCO	1,4-Diazabicyclo[2.2.2]octane
DAPI	4,6-diamidino-2-phenylindole
ATF3	activating transcription factor 3
AP-1	activator protein-1
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ANOVA	analysis of variance
BBB	blood-brain barrier
BSA	bovine serum albumin
BDNF	brain-derived neurotrophic factor
CREB	cAMP response element binding protein
CNS	central nervous system
CTB	cholera toxin subunit B
CSPG	chondroitin sulfate proteoglycan
CNTF	ciliary neurotrophic factor
CRMP-2	collapsing response mediator protein-2
cAMP	cyclic adenosine monophosphate
dpo	days post operation
db-cAMP	dibutyryl-cAMP
DRG	dorsal root ganglion
E-NTPDase	ectonucleoside triphosphate diphosphohydrolase

E-NPPs	ectonucleotide pyrophosphatase
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acidic protein
GDNF	glial-derived neurotrophic factor
GAG	glucosaminoglycan
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPI	glycosylphosphatidylinositol
GAP43	growth associated protein 43
EC50	half maximal effective concentration
HCl	hydrochloric acid
Ig	immunoglobulin
InsP3	inositol triphosphate
IL	interleukin
JAK	Janus kinase
JNK	Jun kinases
LIF	leukemia inhibitory factor
MAG	myelin associated glycoprotein
NGF	nerve growth factor
NCAM	neural cell adhesion molecule
NT	neurotrophin
NgR	Nogo receptor

NDS	normal donkey serum
NGS	normal goat serum
OEC	olfactory ensheathing cell
OMG	oligodendrocyte myelin glycoprotein
$p75^{NTR}$	p75 neurotrophin receptor
PFA	paraformaldehyde
PNS	peripheral nervous system
PB	phosphate buffer
PBS	phosphate buffered saline
pSTAT3	phospho-STAT3
pcJun	phospho-cJun
PLL	poly-l-lysine
PCR	polymerase chain reaction
PSA	polysialic acid
PST	polysialytransferase
PKA	protein kinase A
qPCR	quantitative PCR
RAGs	regeneration-associated genes
SC	Schwann cells
SN	sciatic nerve
SEMA3A	semaphorin-3A
STAT3	signal transducer and activator of transcription 3
SEM	standard error of the mean

TRITC	tetramethyl rhodamine isothyocyanate
TRPC4	transient receptor potential C4
TBS	tris buffered saline
TNF	tumor necrosis factor
UDP	uridine diphosphate
UTP	uridine triphosphate
VNUT	vesicular nucleotide transporter

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CHAPTER 1 - GENERAL INTRODUCTION

1.1 Neuroregeneration in PNS and CNS

The human body is made up of about 60-100 trillion individual cells. To maintain the various functions of this complex organism, there are two controlling systems: the endocrine system and the nervous system. The endocrine system typically functions by synthesizing hormones in an organ and transporting them to the target organ through the bloodstream or lymph. Its operation is analogous to 'wireless communication' in the body. On the other hand, the nervous system employs a system of 'wire communication'; this system transmits signals between different parts of the body through nerve fibres.

Consequently, when there is damage to nerve fibres, this communication is disrupted and needs to be reconnected. The process of regrowth and reconnection of the nerve fibres is called 'neuroregeneration'. The nervous system can be broken down into two systems: the central nervous system (CNS) and the peripheral nervous system (PNS). Neuroregeneration, the body's automatic response following injury, differs between the CNS and PNS in adult mammals (Figure 1-1).

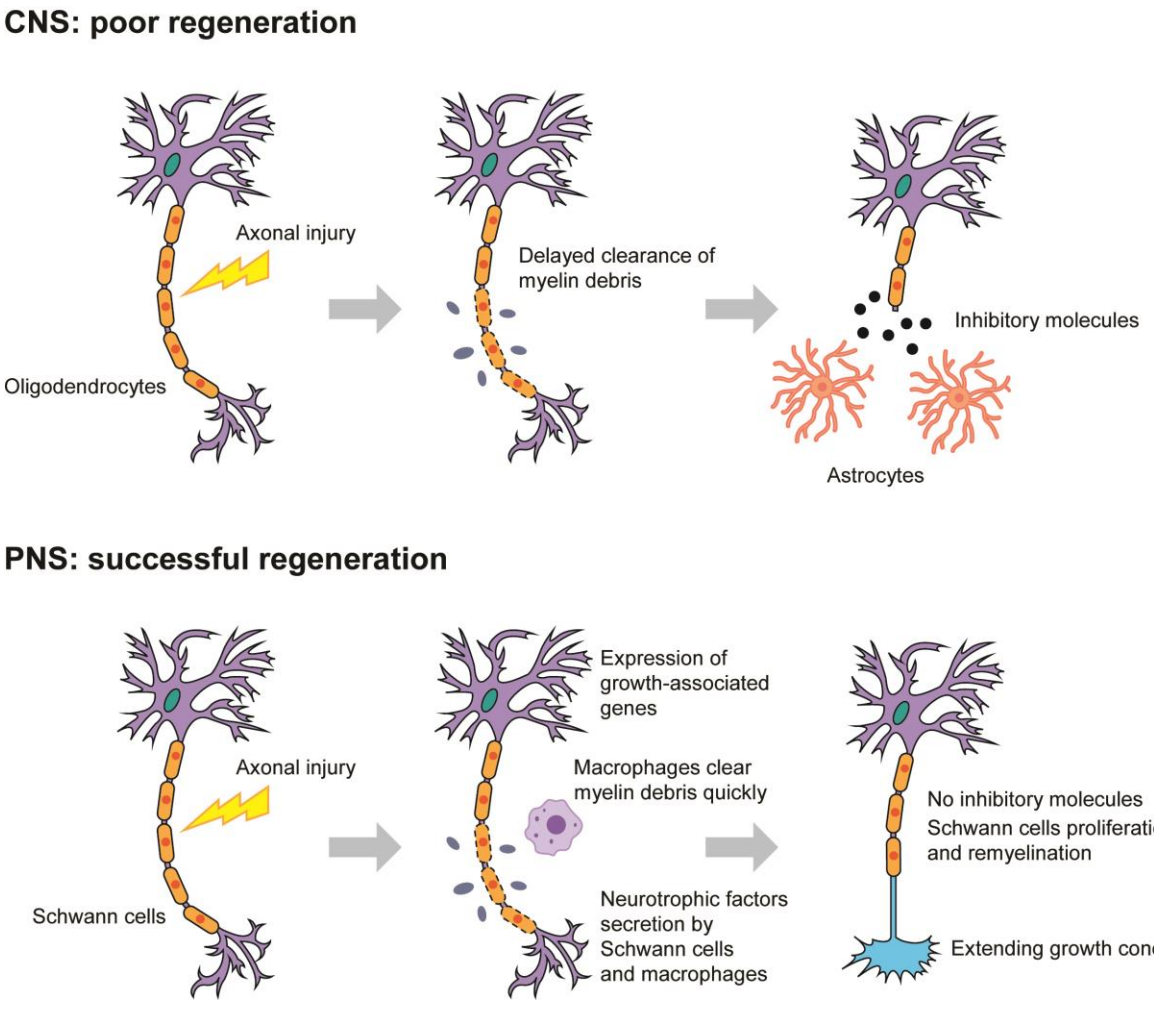


Figure 1-1 Difference between PNS and CNS injury and regeneration.

Within 24-36 hours of a PNS or CNS lesion, self-destructive Wallerian (anterograde) degeneration, including axonal destruction and myelin disintegration, appears in the distal end, while the proximal end reseals the axonal membrane (Fishman and Bittner, 2003, Schlaepfer and Bunge, 1973, Rotshenker, 2011). During Wallerian degeneration, macrophages and microglia are increased in number either by filtration from monocytes in blood or by proliferation (Lu and Richardson, 1993, Priller et al., 2001). However, while myelin and axon debris are cleared quickly by macrophages in interaction with Schwann

cells in the PNS, the process in the CNS is both delayed and far less efficient by microglial cells (George and Griffin, 1994, Vargas and Barres, 2007, Burnett and Zager, 2004).

The next crucial step for regeneration is the formation of the growth cone from the proximal end. However, unlike in the PNS, CNS neurons in adult mammals fail to form growth cone-like structures but instead form a retraction bulb or an end bulb and undergo dying-back away from the lesion (Hill et al., 2001, Li and Raisman, 1995, Bernstein and Stelzner, 1983, Bregman et al., 1989). Even though these injured CNS axons attempt to sprout or regrow, it is only transient and abortive (Bregman et al., 1989, Joosten et al., 1995, Li et al., 1997, Li et al., 1998). It is probable that the extremely limited ability of CNS neurons to regenerate is the result of evolutionary changes to the nervous system in higher animals like mammals since chaotic regeneration in such a highly sophisticated system is likely to be a disaster (Afshari et al., 2009). Adequately promoting CNS regeneration remains one of the most potentially useful challenges for modern medicine, given the numerous problems and suffering experienced by CNS-injured patients.

In PNS, once degeneration events are complete, the only remaining structure is a column of collapsed (un-innervated) Schwann cells called a 'band of Büngner' (Ribeiro-Resende et al., 2009). Axonal sprouts with a growth cone regrow using a band of Büngner as a guide, eventually reaching and reinnervating the target tissue (Ribeiro-Resende et al., 2009, Burnett and Zager, 2004). After reinnervation, the axon matures and cytoarchitecture and function of the neurons are finally restored (Burnett and Zager, 2004). However, incomplete sensory and motor functional recovery might take place when the nerves regrow in a different way and do not successfully reform functional connections (Burnett and Zager, 2004).

1.2 Spinal cord injury and repair strategies

Spinal cord injury is the area in which CNS regeneration is particularly interesting and has been extensively studied. There are over two million people affected by spinal cord injury worldwide (Afshari et al., 2009). The injury can be caused by contusion, compression or laceration, but contusion is the most common type of spinal cord injury (Oudega, 2007). The symptoms or conditions vary, depending on the cause and the severity of the injury, and range from spasticity and paralysis to sensory changes and numbness (Afshari et al., 2009). Spinal cord injury primarily causes loss of cells at the injury site and it initiates inflammatory and cytotoxic responses leading to secondary damage and formation of fluid-filled cysts as well as Wallerian degeneration and demyelination of the axons (Kwon et al., 2002).

Repair strategies for trauma to the spinal cord should include regeneration of injured axons or compensatory growth of new connections from nearby undamaged axons called sprouting (Afshari et al., 2009, Tuszynski and Steward, 2012). The first step of the successful regeneration process involves the sealing of the plasma membrane at the cut end, followed by the disintegration of the cytoskeletons, growth cone formation, regrowth of the axons to the target area and finally synapse formation (Afshari et al., 2009). However, the success rate of regeneration varies, depending on neuronal type, age and distance of the injury site from the cell body: peripheral, embryonic/neonatal neurons or injured axons closer to their cell body regenerate better than central, adult neurons or injured axons far from their cell body (Afshari et al., 2009).

Successful regeneration in the PNS suggests possible strategies to promote axonal regeneration in the CNS (Figure 1-1). Firstly, two strong elements of PNS regeneration are

the neurotrophic-factor-rich permissive environment created by Schwann cells and the lack of inhibitory molecules (Afshari et al., 2009), which is in sharp contrast to the CNS where axons are surrounded by oligodendrocytes and many inhibitory molecules are present: these are external factors to be targeted to manipulate in the CNS. Moreover, there are differences between the CNS and PNS in molecular changes after injury. For example, only in the PNS, axotomy induces regeneration-associated genes Jun and growth associated protein 43 (GAP43) as well as genes related to the JAK/STAT3 pathway (Raivich et al., 2004, Skene, 1989, Qiu et al., 2005): these are the intrinsic factors to additionally be targeted to promote axonal regeneration in the CNS. External factors and intrinsic abilities of axonal regeneration will be discussed in details in the following sections.

1.3 Extrinsic factors of axonal regeneration

1.3.1 Reactive gliosis and glial scar formation

Reactive gliosis is associated with hypertrophic changes and proliferation of the astrocytes, dispersion of the oligodendrocytes, with invasion of meningeal fibroblasts, followed by disruption of the blood-brain barrier (BBB), which ultimately form dense glial scar at the site of injury (Silver and Miller, 2004, Fawcett and Asher, 1999). Cytokines and chemokines, released from inflammatory and immune cells at the site of injury, including interferon-gamma (Yong et al., 1991), TGF-beta (Asher et al., 2000), interleukin-1 (Giulian et al., 1988) and interleukin-6 (Okada et al., 2004, Nakamura et al., 2005) are known to be involved in glial scar formation. Reactive gliosis and glial scar formation are beneficial because they limit further damage and preserve function by setting up a boundary between damaged and non-damaged tissue (Okada et al., 2004, Nakamura et al., 2005, Faulkner et

al., 2004). A study showed that depletion of reactive gliosis resulted in severe demyelination and increased cell death and prevented BBB re-establishment (Faulkner et al., 2004). However, the adverse effect of mature glial scar is that it acts as a physical and chemical barrier to the regrowth of axons preventing reconnection after injury (Windle et al., 1952, Kwok et al., 2008, Niclou et al., 2006).

1.3.2 Myelin inhibitors

Myelin inhibitors are the main inhibitory components within the CNS. Nogo (reticulon 4) was the first identified inhibitory molecule of neurite growth in the CNS and it has been studied extensively to date (Caroni and Schwab, 1988, Spillmann et al., 1998, Chen et al., 2000). There are three isoforms of Nogo depending on differential splicing and promoter usage: Nogo-A, -B and –C (Schwab, 2010). Nogo-A has two known inhibitory domains: Nogo-66 (common to all three isoforms of Nogo) and Nogo-delta 20 (Karnezis et al., 2004, Schwab, 2010). The application of antibodies blocking Nogo-A resulted in the promotion of axonal regeneration and compensatory sprouting as well as marked improvements of functional recovery in spinal cord injured rats and macaque monkeys (Schnell and Schwab, 1990, Fouad et al., 2004, Freund et al., 2006, Zorner and Schwab, 2010). A Phase I clinical trial to test an anti-Nogo-A antibody on acute spinal cord injury patients has been successfully completed and a multicentric, multinational Phase II trial is still in preparation (Blight and Tuszynski, 2006, Zorner and Schwab, 2010).

Myelin associated glycoprotein (MAG) is a transmembrane protein which hampers axonal outgrowth from DRG and cerebellar granule cells (Martini, 1994, Mukhopadhyay et al., 1994, McKerracher et al., 1994). On the other hand, oligodendrocyte myelin glycoprotein (OMG) is a glycosylphosphatidylinositol (GPI)-linked protein which causes

growth cone collapse in many types of neuronal cells (Mikol and Stefansson, 1988, Kottis et al., 2002).

Structurally, Nogo receptors (NgR) contain eight leucine-rich repeat (LRR) domains flanked by the N-terminal and C-terminal cysteine-rich regions (Kobe and Kajava, 2001). The LRR domain is connected to the GPI-anchor via a C-terminal stalk region for membrane attachment (Semavina et al., 2011). The stalk region might be involved in the interactions between NgR and co-receptors such as LINGO1 (Semavina et al., 2011). In addition to NgR1, two other isoforms of the receptor, NgR2 and NgR3, were identified based on amino acid sequence homology and biochemical similarity (Barton et al., 2003, Lauren et al., 2003, Pignot et al., 2003, Klinger et al., 2004, Venkatesh et al., 2005). NgR1 serves as common receptor for the Nogo-66 inhibitory domain, as well as MAG and OMG; NgR2 was known to be a binding partner for MAG (Fournier et al., 2001, Liu et al., 2002, Wang et al., 2002, Venkatesh et al., 2005). Since MAG and OMG act through NgR, manipulation of NgR using anti-NgR antibody, soluble NgR, or dominant-negative NgR could control the inhibitory effects of MAG and OMG as well as Nogo on neurons (Domeniconi et al., 2002, Wang et al., 2002).

1.3.3 Chondroitin sulfate proteoglycans

Proteoglycan is the major inhibitory element in the glial scar produced by astrocytes (Gallo et al., 1987). One type of this is chondroitin sulfate proteoglycans (CSPG). CSPG disrupt the function of integrins, which are key intrinsic factors in axonal regeneration, on the axons and therefore prevent growth cones reacting to the growth-promoting cues (McKeon et al., 1995, Zhou et al., 2006, Smith-Thomas et al., 1994). Interestingly, a recent study identified NgR1 and NgR3 as CSPG receptors, suggesting that there is functional

redundancy among CSPG receptors (Dickendesher et al., 2012). Enzyme chondroitinase ABC, derived from the bacteria *Proteus vulgaris*, can digest sulfated glucosaminoglycan (GAG) chains on CSPG, which is the main proteoglycan inhibitory component (Crespo et al., 2007). Studies have shown that intrathecal treatment of chondroitinase ABC could enhance axonal regeneration and sprouting as well as functional recovery after spinal cord injury (Caggiano et al., 2005, Bradbury et al., 2002).

1.3.4 Semaphorins

Semaphorins are a large family of guidance molecules. The axon repellent role of semaphorin-3A (SEMA3A), produced by meningeal fibroblasts in the glial scar, has been well studied in the field of spinal cord injury (Pasterkamp et al., 2001, Pasterkamp et al., 1998). SEMA3A is suggested to create an exclusion zone for regenerating axons in the dorsal column and that enhancing the intrinsic regenerative response of DRG neurons could elicit only limited effects on the regrowth of fibres (Pasterkamp et al., 2001). Application of a selective SEMA3A inhibitor, SM-216289 (xanthofulvin), has shown a substantially enhanced preservation/ regeneration rate of injured axons and robust myelination, resulting in functional recovery after spinal cord injury (Kikuchi et al., 2003, Kaneko et al., 2006).

1.3.5 Ephs and ephrins

Class A and B ephrins interact with Eph receptors (EPHA1-10 and EPHB1-6) and Eph receptors inhibit axonal extension through their effects on the actin cytoskeleton (Fabes et al., 2006). Some Eph receptors, such as EPHA3, EPHA4, EPHA6, EPHA8 and EPHB3 are found to be upregulated after spinal cord injury (Bundesen et al., 2003, Willson et al., 2002, Miranda et al., 1999). In particular, this injury-mediated upregulation of EPHA4 is related to reactive gliosis and glial scar formation and is inhibitory to axonal regeneration (Fabes et

al., 2006). For instance, EPHA4 knockout mice have shown profound regeneration as well as functional recovery after injury (Goldshmit et al., 2004). Moreover, an EPHA4-blocking peptide showed an absence of retrograde degeneration and axons sprouting up and into the lesion centre after corticospinal tract injury, resulting in recovery in corticospinal tract function (Fabes et al., 2007). Ephs and ephrins are promising targets for the therapeutic tools of spinal cord injury since they modulate integrins and activate RhoA and its inhibitory downstream pathways (Lawrenson et al., 2002, Miao et al., 2000, Yamaguchi et al., 2001).

1.3.6 Polysialic acid

Polysialic acid (PSA) is a cell-surface glycan which binds to neural cell adhesion molecules (NCAM) as a post-translational modification by enzymes polysialytransferases (PST) (Rutishauser, 2008). PSA, with its huge hydrated volume, prevents cell adhesive interactions (Rutishauser, 2008) and renders the glial scar more permissive (Zhang et al., 2007a). PSA-NCAM is highly expressed throughout the embryonic brain, while in adults, it is restrictively present in areas with high degrees of plasticity such as the hippocampus, hypothalamus and olfactory bulb (Finne et al., 1983, Becker et al., 1996, Hoyk et al., 2001, Kiss and Muller, 2001). Induced expression of PSA-NCAM using viruses carrying PST resulted in enhanced axonal sprouting and regrowth in the corticospinal tract (El Maarouf et al., 2006).

1.3.7 Neurotrophic factors

As mentioned above, one of the reasons for successful PNS axonal regeneration is the ability of Schwann cells to release neurotrophic factors. The CNS has also been shown to react to neurotrophic factors when induced exogenously. The results from genetically

engineered neurotrophic-factor-secreting cells or matrices that contains neurotrophic factors are promising in promoting the regrowth and sprouting of CNS axons (Lu and Tuszynski, 2008). Neurotrophic factors which enhance axonal regeneration include glial-derived neurotrophic factor (GDNF) (Ramer et al., 2000, Blesch and Tuszynski, 2003), nerve growth factor (NGF) (Ramer et al., 2000), brain-derived neurotrophic factor (BDNF) (Kobayashi et al., 1997) and neurotrophin (NT)-3-5 (Ramer et al., 2000, Kobayashi et al., 1997, Schnell et al., 1994, Blesch et al., 2004). Artemin, a GDNF family member, is an example of a specific-acting neurotrophic factor. It has shown potent neurotrophic effects on injured C-fibres (Bennett et al., 2006) and virus-mediated artemin delivery helped overcome myelin inhibitions resulting in significant motor function recovery after injury (Zhou et al., 2009). The use of neurotrophic factors is considered one of the most important components in combined therapeutic approaches to promote axonal regeneration (Afshari et al., 2009).

1.3.8 Inflammatory cells

Although inflammation was traditionally regarded as harmful to the regenerative process due to its neurotoxicity mediated by microglial cells (Block et al., 2007), there is growing evidence that inflammation can actually be beneficial. That is, activated microglia/macrophages at the site of injury can remove debris and secrete neurotrophic factors such as BDNF and GDNF resulting in increased axonal regeneration (Hashimoto et al., 2005, Dougherty et al., 2000). In the acute phase of injury, CSPG, the main inhibitory component of glial scar, can directly activate microglia/macrophages to modulate neurotrophic factor secretion (Rolls et al., 2008). Accordingly, acute removal of CSPG after injury can be damaging as it interrupts these immune-related actions, while delayed removal of CSPG for two days has been shown to preserve the beneficial aspects of

inflammation (Rolls et al., 2008). Therefore, using inflammation for regeneration requires a sensible approach in respect of the temporal and dynamic nature and mechanisms of axonal injury and repair (Afshari et al., 2009).

A variety of inhibitory or stimulatory extrinsic factors for regeneration were discussed above. To conclude, the CNS microenvironment has to be made more permissive either by promoting positive cues or by inhibiting negative cues (Afshari et al., 2009).

1.4 Intrinsic ability of CNS regeneration

Any manipulation of external factors to make the CNS environment more permissive is no use if the axons do not attempt to regenerate. In fact, the simple deletion of inhibitory molecules by pharmacological means or genetic modification has only resulted in limited sprouting, but not long distance axonal regeneration (Yiu and He, 2006, Lee et al., 2009). It is possible that key inhibitory factors have not yet been identified, but it is still more likely that the intrinsic growth mechanisms are largely involved in the regeneration process. The intrinsic regenerative abilities of CNS neurons and possible strategies to increase or restore such abilities will be further explained in this section.

1.4.1 Responses to nerve injury

To closely examine the regenerative responses of injured central axons will be beneficial to better understand the underlying mechanism of intrinsic regenerative abilities. Following axotomy, sensory neurons in DRG and motor neurons in the spinal cord or brainstem undergo significant morphological and molecular changes (Richardson et al., 2009).

Histologically, axotomy-induced acute changes in neuronal bodies, referred as ‘chromatolysis’ including Nissl substance dispersal, nucleus displacement and cell body swelling, were observed more than a century ago (Richardson et al., 2009). In the 1970s, following advances in microscopic techniques, more refined observation such as the disassociation of polyribosomes with lamellae of the rough endoplasmic reticulum were made (Lieberman, 1971). In the long-term, the cell bodies of the CNS neurons undergo atrophic changes such as reduced cell volume and dendritic arborisation, in sharp contrast to PNS neurons where hypertrophic changes as well as increased metabolism and protein synthesis occur (Lieberman, 1971, Liu et al., 2011). Some neurons even undergo cell death especially in the case of proximal axotomy (Richardson et al., 2009).

Molecular changes including neurotrophic factors, inflammatory cytokines, transcription factors and cytoskeleton associated protein were observed after axotomy. Neuropoietic cytokines such as CNTF, IL6 and LIF activate the gp130 receptor, which recruits JAK and phosphorylates STAT3 so that it can be dimerized and translocated into the nucleus. Following nerve injury, the release and synthesis of CNTF and LIF by Schwann cells (Sendtner et al., 1992, Subang and Richardson, 2001) and IL-6 by neurons is increased (Murphy et al., 1995). Furthermore, phosphorylation and translocation of STAT3 into the nuclei of the sensory and motor neurons is significantly increased (Qiu et al., 2005, Schwaiger et al., 2000). Studies have shown that STAT3 is required in axonal regeneration after peripheral injury in vitro and in vivo (Liu and Snider, 2001, Qiu et al., 2005) and administration of an inducible form of STAT3 into neurons resulted in increased neurite outgrowth (Miao et al., 2006). Also, BDNF, which prevents atrophy of the neurons and promotes axonal regeneration when administered near cell bodies (Kobayashi et al., 1997), is newly synthesized in DRG neurons after axotomy (Michael et al., 1999).

Inflammatory cytokines such as interleukin-1 (IL-1) and tumour necrosis factor-alpha (TNF-alpha) are thought to be involved in stimulating axonal regeneration after axotomy either through direct action or through promoting synthesis of the nerve growth factor in non-neuronal cells (Lindholm et al., 1987, Miao et al., 2008). Cytokine producing macrophages can increase in number at the site of injury and in the DRG after axotomy (Lu and Richardson, 1993). After nerve transection, activation of Jun kinases (JNK) including phosphorylation and translocation into nuclei occurs, followed by the activation of cJun including phosphorylation and increased transcriptional activity. The activated cJun then forms dimers with Fos to create the transcription factor activator protein-1 (AP-1) (Kenney and Kocsis, 1998, Lindwall and Kanje, 2005). IL-1 and TNF-alpha as well as other possible factors contribute to these phosphorylation cascades (Richardson et al., 2009). cJun has been shown to be involved in axonal regeneration, supported by the evidence that deletion of cJun in neurons resulted in impaired axonal regrowth (Raivich et al., 2004).

However, information about the initial molecule or signal which triggers the synthesis and the release of neurotrophic factors or inflammatory cytokines after axotomy is lacking (Richardson et al., 2009). There are only certain assumptions that a response to the danger signals conveyed by the immune system, in which ligands of toll-like receptors such as heparan sulfate are involved, might be implicated (Gallucci and Matzinger, 2001).

Expression of regeneration-associated genes/molecules increases after nerve injury and works in favour of increased growth propensity. Some of these effector molecules also act on the growth cones and influence cytoskeleton remodelling which mediates axonal elongation (Richardson et al., 2009). In the growth cone, calcium as well as Rho and cAMP also play a key role in regulating cytoskeletal molecules: calcium and cAMP are stimulatory mediators, while Rho acts as an inhibitory mediator of the signalling pathways

(Gomez and Zheng, 2006, Hall and Nobes, 2000, Han et al., 2007). It has been shown that GAP43 promotes neurite outgrowth, neural plasticity and regeneration by modulating phosphatidylinositol 4,5-bisphosphate at the plasmalemmal rafts (Laux et al., 2000). The transient receptor potential C4 (TRPC4) channel is known to increase in rat DRG neurons after axotomy and is necessary for neurite outgrowth. Calcium entering the cytoplasm through the activation of the TRPC4 channel could bind calmodulin and interact with GAP43, which could free cofilin, a key enzyme in actin remodelling, to interact with actin, resulting in actin remodelling (Wu et al., 2008). In addition, collapsing response mediator protein-2 (CRMP-2), a microtubule associated protein, can interact with tubulin heterodimers to promote microtubule configuration at the wrist of the growth cone and this process is involved in the growth cone mobility (Arimura and Kaibuchi, 2007).

1.4.2 Conditioning lesion

Pseudounipolar DRG neurons are unique in their structure in that they have a central axon that enters into the spinal cord and a peripheral axon that innervate peripheral targets with one cellular body. The DRG is also an interesting and unique model for regeneration because its two branches of peripheral and central axons react differently upon axotomy even though they share the same cell body. The peripheral branch regrows effectively after injury but the central branch struggles to regrow and show only abortive sprouting. However, a series of innovative studies have revealed that we can actually use the benefit of the structure to understand the mechanism of the intrinsic abilities of regeneration and also to promote CNS regeneration.

An initial injury to the peripheral nerve, termed a ‘conditioning lesion’, causes the priming of neurons that significantly enhances regeneration upon the subsequent injury at

either the PNS (McQuarrie and Grafstein, 1973) or CNS (Richardson and Issa, 1984, Neumann and Woolf, 1999). Even though this rule applies equally to both the PNS and CNS, it is nevertheless particularly important in CNS injuries due to the CNS's lack of regenerative ability. The following paragraphs are the brief history of the conditioning lesion phenomenon.

The concept of the conditioning phenomenon was first described by McQuarrie and Grafstein, who showed enhanced axonal outgrowth following a previous nerve injury in adult mice (McQuarrie and Grafstein, 1973). The rate of axonal regeneration after a nerve cut (termed a 'testing lesion') was 27% faster than normal if the nerve had been crushed (termed a 'priming lesion' or 'conditioning lesion') two weeks before excision. In fact, both regenerating and crushed nerves were of the PNS; the sciatic nerve, tibial and peroneal nerves, respectively. However, the authors pointed out that since the testing lesion was well distant from the conditioning lesion, local changes in the nerve from the conditioning lesion would not have affected the axonal outgrowth at the testing lesion site (McQuarrie and Grafstein, 1973, McQuarrie et al., 1977). They suggested that the growth promoting effect of the conditioning lesion is probably the result of the central changes at the level of the neuronal cell body (McQuarrie and Grafstein, 1973, McQuarrie et al., 1977), opening up the possibility of further studies in the area. In addition, they revealed that a conditioning lesion can also accelerate the motor axonal outgrowth and the 'conditioning lesion effect' is confined to the ipsilateral branch of axons which had received the conditioning lesion (McQuarrie, 1978).

Richardson and his colleague brought the conditioning lesion to the field of the CNS. They paid particular interest to the L4 and L5 DRG, which contain numerous large neurons projecting to both the CNS and PNS, centrally to the brain stem and peripherally to

the sciatic nerve. They showed that long spinal axons of primary sensory neurons are 100 times more likely to regenerate into the peripheral nerve graft at the lesion site if their peripheral axons are also cut (Richardson and Issa, 1984). In other words, lesion of a peripheral nerve significantly enhanced the regenerative capacity of the central axons of corresponding DRG neurons. They also confirmed that a conditioning lesion does not affect the axonal regrowth of contralateral DRG. In 1999, Neumann and Woolf used this model to demonstrate whether the conditioning lesion could promote axonal regeneration in the spinal cord even in the absence of permissive peripheral nerve graft (Neumann and Woolf, 1999). As a result, rats with a sciatic nerve conditioning lesion showed substantial regrowth of axons into and beyond the lesion site after dorsal column transection.

Later, the 'conditioning lesion' was classified into two separate forms according to the timing of the injury: pre-conditioning and post-conditioning. If lesion of peripheral axons is performed before the central lesion, it is called 'pre-conditioning'; if peripheral lesion takes place after the central injury, it is termed 'post-conditioning'. From a therapeutic point of view, post-conditioning is more clinically relevant since it can be used as a potential therapeutic model for the spinal cord injury and repair.

The time window of the central axonal regeneration on peripheral conditioning lesion is not yet completely understood. It has been reported that conditioning lesions promote CNS regeneration only when applied prior to or at the time of injury, but not after a central injury (Neumann and Woolf, 1999, Neumann et al., 2005). However, it has been assumed that a propensity for regeneration of sensory neurons persisted for at least 1 month after injury (Richardson and Issa, 1984) and transcriptional changes occurring in DRG after pre-conditioning and post-conditioning were practically identical (Kadoya et al., 2009). The decline in regeneration after post-conditioning might be due to either environmental

changes at the lesion site or post-transcriptional neuronal responses (Blesch et al., 2012, Richardson and Issa, 1984, Ylera et al., 2009). Recent evidence shows that conditioning lesions remain effective when applied up to 4 weeks (Blesch et al., 2012), 8 weeks (Ylera et al., 2009) or even 15 months (Kadoya et al., 2009) after CNS injury, when combined with other therapies such as NT-3 delivery, cell grafts or interventions reducing the glial scar. The expansion of the time window of the conditioning lesion might open up possibilities of it being used as a therapeutic tool for chronically CNS-injured patients. However, it is not clinically practical to injure nerves to induce CNS regeneration. Therefore, to understand the underlying mechanisms could provide more suitable means/agents to mimic conditioning lesions for enhanced CNS regeneration.

Even though it has been nearly four decades since the conditioning lesion phenomenon was observed, the underlying mechanism is still not fully characterised. Changes in the neuronal cell body are mainly credited for the growth promoting effects of the conditioning lesion. Due to extensive studies to identify the transcription factors, regeneration-inducing genes and signalling pathways involved in stimulating axonal regeneration after conditioning lesions, it is now known that upregulation/elevation of CNTF (Green et al., 2004), IL-6 (Cafferty et al., 2004, Cao et al., 2006), LIF (Cafferty et al., 2001), STAT3 (Qiu et al., 2005), activating transcription factor 3 (ATF3) (Seijffers et al., 2006, Seijffers et al., 2007), cJun (Broude et al., 1997), GAP43 (Skene, 1989), arginase I (Cai et al., 2002), cyclic AMP (cAMP)-related pathways (Cai et al., 2001, Cai et al., 2002, Lu et al., 2004, Neumann et al., 2002) and cAMP response element-binding protein (CREB) (Gao et al., 2004) are involved in conditioning lesion.

Delivery of CNTF to the DRG by a lentivirus vector mimicked the effects of the conditioning lesion on the regrowth of injured dorsal root axons (Green et al., 2004). Also,

IL-6 and LIF are considered as important cytokines for the conditioning lesion since conditioning lesion-induced spinal axonal regeneration failed in IL-6 (Cafferty et al., 2004) and LIF (Cafferty et al., 2001) knockout mice. The JAK/STAT3 pathway, through which neuropoietic cytokines such as LIF, CNTF and IL-6 are mediated, is involved in promoting axonal regeneration after a conditioning lesion. Transcription factor STAT3 activation is considered to be necessary for the increased growth capacity of DRG neurons and enhanced spinal axonal regeneration after a conditioning lesion (Stone et al., 2004). Intrathecal delivery of IL-6 to DRG mimicked the conditioning lesion's effects and overcame myelin inhibition after dorsal column injury. However, it was blocked by the inhibition of JAK/STAT3 pathway (Cao et al., 2006).

Other transcription factors which drive the changes in gene expression that increase the intrinsic growth state of 'conditioned' sensory neurons are also involved. ATF3 is strongly upregulated after a conditioning lesion and delivery of ATF3 to DRG neurons using viral vectors enhanced neurite outgrowth (Seijffers et al., 2006). cJun is also upregulated upon the conditioning lesion (Broude et al., 1997) and known to be involved in axonal regeneration as described above (Raivich et al., 2004).

As a conditioning lesion leads DRG neurons to an enhanced growth status, this can be reflected in the upregulation of regeneration-associated genes such as GAP43 in DRG neurons (Schreyer and Skene, 1993, Skene, 1989). In rat DRG, induction of GAP43 mRNA starts between 1 and 2 days after peripheral nerve injury (Skene, 1989) and immunohistochemical studies revealed that GAP43 in DRG neurons is upregulated after peripheral injury, but not after central injury (Schreyer and Skene, 1993). Also, GAP43 is known to be extremely abundant in growth cones, comprising around 1% of the total

protein in growth cones membranes (Skene et al., 1986). These suggest that GAP43 might be essential for axonal growth.

Cyclic AMP (cAMP) is an essential signalling molecule which plays an important role in axonal regrowth. cAMP level increase in the cell body is one of the distinctive changes after injury and is known to be deeply involved in the conditioning lesion (Meyer-Franke et al., 1998). Direct cAMP injection has been reported to replicate the conditioning lesion effects (Lu et al., 2004, Neumann et al., 2002). Elevating cAMP level, either with dibutyryl-cAMP (db-cAMP) or by prior exposure to neurotrophins, helps neurons overcome myelin inhibitors such as MAG (Cai et al., 1999, Gao et al., 2003). cAMP activates protein kinase A (PKA) and consequently upregulates the transcription factor CREB, which mediates the effects of neurotrophic factors (Cai et al., 1999, Gao et al., 2003). cAMP also can induce stronger regenerative responses in spinal cord injured models (Qiu et al., 2002a, Qiu et al., 2002b). cAMP also raises axonal regenerative ability by supporting the translocation of growth factor receptor TrkB, from intracellular stores to the plasma membrane (Meyer-Franke et al., 1998). However, a recent study showed that cAMP-mediated mechanisms account for only part of the effects on CNS regeneration (Blesch et al., 2012), suggesting that concerted activation of several integrated mechanisms/pathways may be required to initiate the intrinsic regenerative abilities of adult DRG neurons after injury. Also, cAMP analogues increased neurite outgrowth on postnatal DRG but had no effect on adult DRG (Blesch et al., 2012, Murray and Shewan, 2008).

Since the decreased intrinsic regenerative ability of adult neurons is considered a major contributor to the failure of CNS regeneration (Sun and He, 2010), studies on the conditioning lesion remain invaluable.

1.4.3 Other intrinsic factors

Rho is a Ras-related GTPase which is known to inhibit neurite outgrowth (Yamaguchi et al., 2001). Studies have shown that inhibition of the Rho pathway results in promoting neurite and axonal growth in DRG (Borisoff et al., 2003) and primary cortical neurons (Dergham et al., 2002). The Rho pathway is involved in the effects of inhibitory molecules such as myelin (Fournier et al., 2003), CSPG (Monnier et al., 2003) and ephrins (Wahl et al., 2000). Application of C3 and Y27632, common inhibitory agents of the Rho pathway, promoted axonal regeneration and motor function recovery after dorsal crush injury in mice (Dergham et al., 2002). Similar results were obtained using a high dose of Y27632 in dorsal column transection models in rats (Chan et al., 2005). Rho inhibitor Cethrin was used in clinical trials on acute spinal cord patients (Blight and Tuszynski, 2006).

Lipid rafts are lipid-rich domains; cholesterol-rich regions are important in growth cone guidance, and ganglioside-rich regions are involved in axonal elongation (Guirland et al., 2004). Gangliosides, including GD1a, GT1b and GM1, have been more actively studied and different gangliosides are known to have different effects in axonal regeneration. GD1a and GT1b are functional nerve cell surface ligands of MAG, and therefore inhibitory to axonal regeneration. In contrast, GM1 ganglioside promotes axonal regeneration. Application of GM1 antibody inhibited neurite outgrowth (Wu et al., 1994, Spirman et al., 1984) and sialidase, an enzyme which converts GD1a and GT1b to GM1, promoted spinal axonal growth in vivo (Yang et al., 2006).

Integrins are transmembrane receptors which are considered to be key intrinsic factors in axonal regeneration. Integrins, a family of alpha and beta heterodimeric receptors, mediate interactions between cells or between cell and substratum (Neugebauer and

Reichardt, 1991). After axonal injury, several types of integrin subunits, including alpha6, alpha7 and beta1, are upregulated in DRG neurons (Wallquist et al., 2004). Alpha7 integrin is known to be involved in the preconditioning lesion and alpha7 knockout mice have shown impaired axonal regeneration (Ekstrom et al., 2003). Furthermore, overexpression of alpha9 integrin in rat DRG using lentiviruses resulted in enhanced regeneration and functional recovery after cervical dorsal rhizotomy or dorsal column crush (Andrews et al., 2009).

1.5 Therapeutic strategies for CNS repair

There have been numerous studies to improve the treatment of spinal cord injury and combinatory treatments have achieved greater axonal regrowth than therapies applied in isolation (Lu and Tuszynski, 2008). This is due to the fact that the mechanisms of spinal cord injury and repair are complicated and a single therapeutic agent with a single mechanism of action is unlikely to cover the all aspects of the strategies to overcome spinal cord injury. The main strategies of a combined therapy would be: (1) to make the external environment more permissive by promoting supportive factors of regeneration and removing inhibitory factors of regeneration, (2) to enhance the intrinsic capacity of neurons for regrowth, and (3) to increase the plasticity of the neurons (Afshari et al., 2009, Cafferty et al., 2008, Blesch and Tuszynski, 2009).

The transplantation of cells, such as Schwann cells, olfactory ensheathing cells (OECs) and neural stem cells, to provide the path for axonal regeneration is frequently used as a part of combined therapy (Guo et al., 1999, Shuster et al., 1999, Vulchanova et al., 1998). Movement rehabilitation after spinal cord injury could also be a component of

combinatory therapy for successful functional recovery (Shuster et al., 2000, Stone et al., 1999). However, not all combined therapies are synergic (Vulchanova et al., 2001) and the effective time window of each treatment has to be taken into account. There should be more studies investigating all possible combined therapeutic approaches and the best combinations, which elicit synergic effects, for the better treatment of spinal cord injury.

Delivery methods also need to be considered. Since systemic delivery of neurotrophic factors can cause severe adverse effects by stimulating unintended organs (Ettinger et al., 2003), local delivery is necessary. So far, gene therapy is the ideal way for local delivery of either neurotrophic factors or transcription factors. Using viral vectors, synthesis of required protein can persist for a protracted period of time ranging from several weeks to months and a high local concentration of the protein of interest can be achieved (Wu et al., 2007). Moreover, these expressions can be restricted to specific targets (cells) or initiated only after taking an agent orally (Richardson et al., 2009). Further modification of viral vectors, such as intercorporating small proteins which are transported retrogradely in axons, makes it possible to inject viral vectors into the nerve or muscle rather than in the vicinity of the neurons (Azzouz et al., 2004, Federici et al., 2007). However, safety issues regarding the use of viral vectors still remain and gene therapy is currently used only in life-threatening situations. Further advances in the study in this field will hopefully open up the possibilities of its broader application in the near future.

CNS Regeneration strategies mentioned in this chapter are summarized in Figure 1-2.

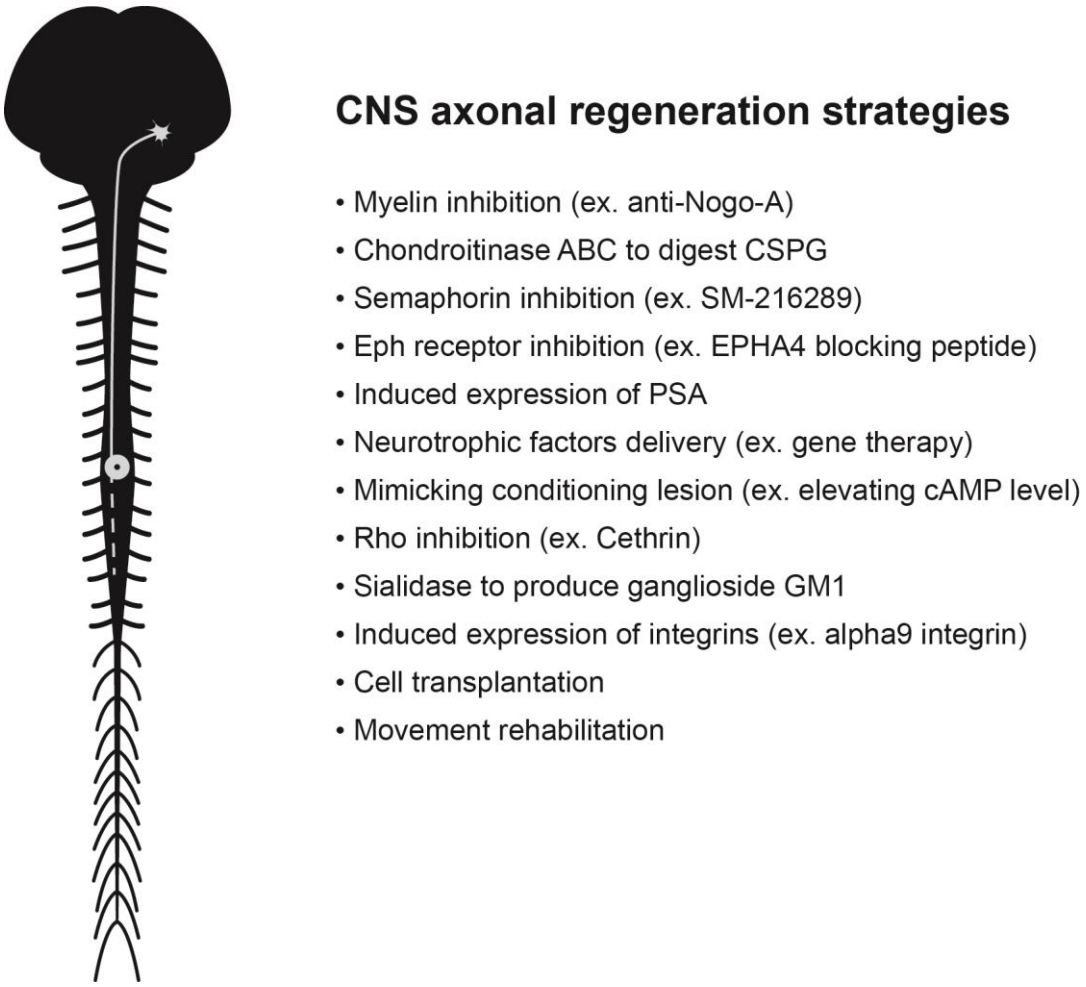


Figure 1-2 Strategies to promote CNS axonal regeneration.

1.6 ATP

1.6.1 Extracellular ATP as a signalling molecule

ATP (Figure 1-3) is a ubiquitous endogenous substance which is well-known for its intracellular role as a fundamental source of energy for all living cells. ATP is also an important molecule in extracellular purinergic signalling (Burnstock, 2007b). Since 1972 when purinergic signalling was first introduced (Burnstock, 1972), there have been a number of studies to uncover its role in the nervous system. It is now known that ATP

works as either a sole transmitter or as a co-transmitter in the CNS and PNS (Burnstock, 2007a). Also, ATP acts as a fast excitatory neurotransmitter as well as a long-term neurotransmitter with its trophic roles in cell proliferation, development and growth (Zimmermann, 2006, Abbracchio and Burnstock, 1998). Neurons have been considered to be the only source of neuromodulatory ATP in the CNS. However, recent studies demonstrate that glial cells also release ATP, suggesting that glial cells could modulate neuronal activity by activating ATP receptors (Cotrina et al., 1998, Wang et al., 2000, Newman, 2001, Newman, 2003, Cao et al., 2013).

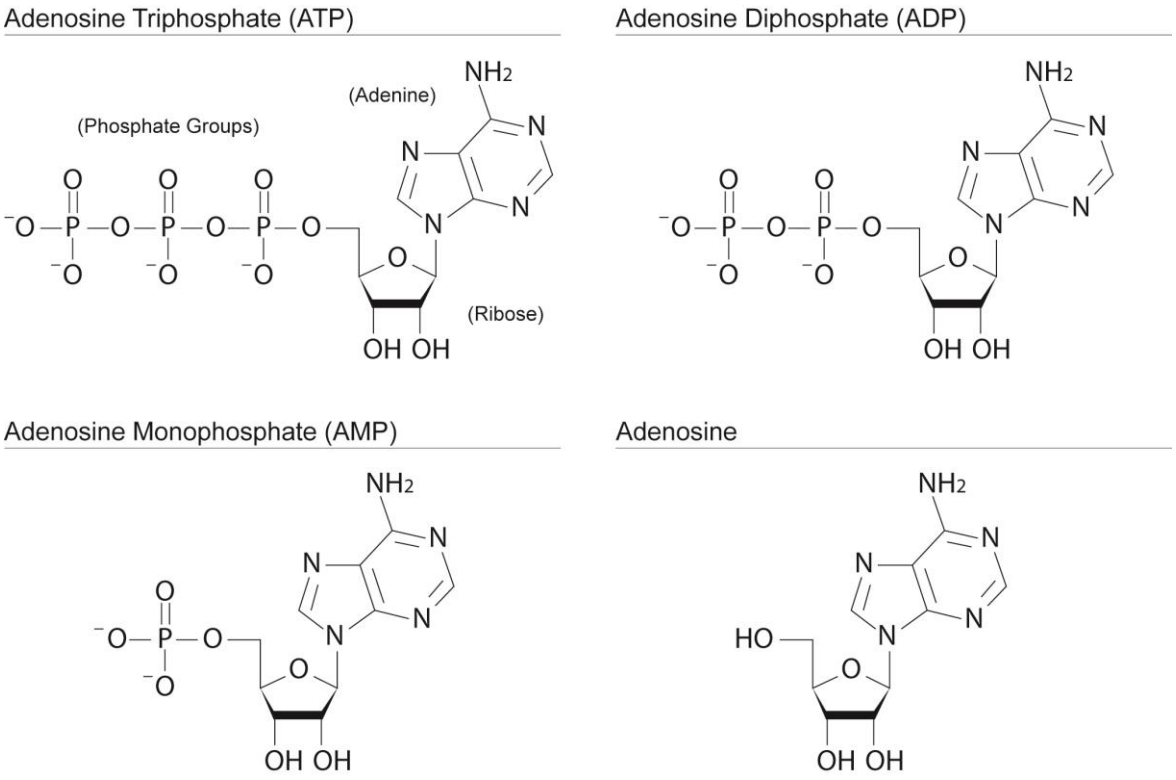


Figure 1-3 Structure of ATP and its metabolites.

1.6.2 Storage, release and degradation of ATP and other nucleotides

ATP and other nucleotides are accumulated and stored in the synaptic and secretory vesicles, which are mediated by a Cl^- dependent vesicular nucleotide transporter (VNUT) (Sawada et al., 2008). It is assumed that almost all synaptic and secretory vesicles have ATP and that ATP is co-stored and co-released with other neurotransmitters such as acetylcholine, noradrenaline, gamma-aminobutyric acid (GABA), dopamine or glutamate (Abbracchio et al., 2009).

There is evidence of three ways to release ATP to date: 1) exocytic vesicular release (Pankratov et al., 2006, Pankratov et al., 2007, Zhang et al., 2007c); 2) transporters such as ATP-binding cassette transporters (Bodin and Burnstock, 2001); or 3) channels such as connexin or pannexin channels (Scemes et al., 2007), volume-regulated chloride channels (Sabirov and Okada, 2005), and P2X7 receptors (Lazarowski et al., 2003). For example, exocytosis of ATP has been implicated in neurons (Pankratov et al., 2007), Schwann cells (Liu and Bennett, 2003) and astrocytes (Zhang et al., 2007c), hemichannels have been found in neurons (Thompson et al., 2006) and anion transporters have been implicated in Schwann cells (Liu and Bennett, 2003).

Upon release, ATP hydrolyses rapidly by degradating enzymes ectonucleotidases; such as ectonucleoside triphosphate diphosphohydrolases (E-NTPDases), ectonucleotide pyrophosphatase (E-NPPs) and alkaline phosphatases or ecto-5′-nucleotidase (Abbracchio et al., 2009). E-NTPDases and E-NPPs hydrolyse ATP and adenosine diphosphate (ADP) to adenosine monophosphate (AMP), and ecto-5′-nucleotidase further breaks down AMP to adenosine (Abbracchio et al., 2009). Also, there are pathways to interconvert the originally released ligands. Interconverting enzymes, such as ectonucleoside diphosphate kinase and

adenylate kinase, work for nucleotide rephosphorylation and extracellular synthesis of ATP (Abbracchio et al., 2009). Adenosine is generally generated by the degradation of ATP, but it is suggested that some neurons or astrocytes might release adenosine directly (Wall and Dale, 2007).

1.6.3 Physiology and pathophysiology of ATP

Upon activation, ATP mediates excitatory postsynaptic currents in the CNS and works as an excitatory or inhibitory neurotransmitter in the smooth muscles (Burnstock, 2007a, North and Verkhratsky, 2006). In addition, ATP is a gliotransmitter and ATP released from astrocytes mediates calcium waves (Cotrina et al., 2000, Schipke et al., 2002). ATP regulates astrogliosis or microgliosis with the control of migration and proliferation of astrocytes or microglial cells, respectively (Abbracchio and Ceruti, 2006, Pocock and Kettenmann, 2007). ATP is also implicated in neuronal-glial and glial-glial signalling (Fields and Burnstock, 2006). ATP (and adenosine) have neuroprotective effects to preserve neurons and facilitate recovery involving the release of neurotrophic factors and neuropoietic cytokines as well as the activation of neuritogenesis (Abbracchio and Ceruti, 2006). Furthermore, ATP is involved in neural development and neuroregeneration (Zimmermann, 2006, Stone et al., 1998). ATP plays an important role in pain perception (Gerevich and Illes, 2004) as well as in chronic neuropathic pain conditions (Todd and Robitaille, 2006). In addition, ATP is involved in special senses such as vision (Burnstock, 2007a), hearing (Housley et al., 2006, Lee and Marcus, 2008), smell (Gayle and Burnstock, 2005) and taste (Huang et al., 2007).

1.7 Purinergic receptors

Separate membrane receptors for adenosine (P1 receptors) and ATP (P2 receptors), called purinergic receptors, were first formally recognized by Burnstock in 1978 (Burnstock, 1978). P1 purinergic receptors recognise adenosine and P2 purinergic receptors recognise mainly ATP, ADP, uridine triphosphate (UTP) and uridine diphosphate (UDP) (Abbracchio et al., 2009). P2 receptors can be further subdivided into two groups depending on the mechanism of their actions and pharmacology: ionotropic P2X receptors which form cationic ligand-operated channels; and metabotropic P2Y receptors, which are involved in G protein activation (Abbracchio and Burnstock, 1994, Fredholm et al., 1994, Abbracchio et al., 2009, Burnstock and Kennedy, 1985).

Intercellular purinergic signalling have been suggested to appear early in evolution, and is consequently a common route for communication between cells (Abbracchio et al., 2009). Purinergic receptors are thought to be the most abundant receptors in all living creatures including mammals, and can therefore be found in all kinds of tissues/cells and widely expressed in both neuronal and non-neuronal cells in the nervous system (Burnstock and Knight, 2004, Abbracchio et al., 2009)

1.7.1 Adenosine and P1 adenosine receptors

Adenosine is a ubiquitous modulator with its own biological functions and can act via specific adenosine receptors (Gerwins and Fredholm, 1992). Adenosine has various roles in the CNS including modulation of neural and glial cell functions and neural-glial signalling as well as neural development (Fellin et al., 2006, Dare et al., 2007). In addition, adenosine plays an important part in the immune system and is also involved in pathologic conditions such as neurodegenerative diseases, psychiatric disorders and epilepsy (Boison, 2008).

Hitherto four adenosine receptors (A1, A2a, A2b and A3) have been cloned in mammals (Ralevic and Burnstock, 1998, Burnstock, 2007b). All P1 receptors are G-protein coupled receptors: A1 and A3 couple to Gi/o protein inhibiting cAMP production and A2a and A2b bind to Gs protein stimulating cAMP production (Ralevic and Burnstock, 1998).

1.7.2 P2X receptors

To date six homomeric (P2X1, P2X2, P2X3, P2X4, P2X5 and P2X7; P2X6 subunits do not oligomerize with each other but remain as unassembled monomers) and six heteromeric (P2X1/2. P2X1/4, P2X1/5, P2X2/3, P2X2/6 and P2X4/6) P2X receptors have been identified in mammals (Nicke et al., 1998, Roberts et al., 2006, Barrera et al., 2005). P2X7 receptors are activated at 100-1000 µM of ATP while other P2X receptors have half maximal effective concentration (EC50) of approximately 1-10 µM of ATP (Abbracchio et al., 2009). Upon ATP binding, P2X receptors which are ligand-gated cation channels open pores to Na^+, K^+ and Ca^{2+} (Abbracchio et al., 2009).

Although all P2X receptors are expressed in neurons, their expression patterns differ from site to site. Peripheral neurons mainly express P2X2/3 and P2X3 which are involved in pain and temperature sensitivity (Pankratov et al., 2003), while P2X receptors expression in central neurons are mosaic which allows the variability of ATP-mediated excitatory synaptic currents (Pankratov et al., 2003). Among glial cells, P2X1/5 were found in astrocytes (Lalo et al., 2008) and P2X4 in microglial cells (Tsuda et al., 2003). Also, P2X1 and P2X5 were suggested to be present in Schwann cells (Todd and Robitaille, 2006). P2X receptors trigger calcium influx in the CNS which then modulates neurotransmitter release (Sperlagh et al., 2007) and P2X7 is suggested to be involved in the induction of apoptosis via sustained calcium influx into the cells (Ferrari et al., 1999).

1.7.3 P2Y receptors

Eight homomeric P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14) have been cloned so far from mammalian species. P2Y1, P2Y11, P2Y12 and P2Y13 exhibit sensitivity to the adenine nucleotides ATP/ADP, P2Y2, P2Y4 and P2Y6 to uracil nucleotides UTP/UDP, P2Y14 to UTP-glucose, or P2Y2 to both adenine and uracil nucleotides. P2Y receptors can be further divided by two subgroups. The first subgroup of P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 mainly couple to Gq/G11 protein activating phospholipase C and the second subgroup of P2Y12, P2Y13, P2Y14 couple to Gi/o or Gs protein activating or inhibiting adenylyl cyclase (Abbracchio et al., 2006, Fischer and Krugel, 2007).

Although classical models forecast that P2Y receptors exist in monomer form, the existence of homomeric P2Y receptors, or heteromeric P2Y receptors either with other P2Y receptors or with adenosine receptors was also suggested (Ecke et al., 2008, Fischer and Krugel, 2007). Also, it is noteworthy that P2Y signalling is agonist-specific. For example, P2Y11 increases cytosolic calcium, cAMP and phospholipase C/inositol triphosphate (InsP3) when activated by ATP, while cAMP and InsP3 are not increased when activated by UTP (Arvidsson et al., 1995).

P2Y receptors appear very early in development and are widely spread throughout the CNS on both neuronal and non-neuronal cells (Abbracchio et al., 2009). They are involved in physiological states such as the control of transmitters release and the calcium wave generation in astroglial cells, as well as in pathological conditions such as neurodegenerative diseases (Fischer and Krugel, 2007). However, the role of each individual P2Y receptor is still not fully understood (Fischer and Krugel, 2007).

1.8 Working theory and hypothesis

Under normal conditions, the cytoplasmic ATP level is in the millimolar range (10mM) (Sperlágh and Vizi, 1996). The cytoplasm of most neurons contains 2-5mM ATP, and up to 100mM ATP is stored in synaptic vesicles (Burnstock, 2007a). After nerve injury, which causes cellular damage, the ATP stored in neuronal and non-neuronal cells is likely to be released into the extracellular space. It has been suggested that ATP can be released upon mechanical damage or shear stress by many different types of cells in both physiological and pathophysiological states (Dubyak, 2006, Bodin and Burnstock, 2001, Lazarowski et al., 2003, Schwiebert et al., 2003, Burnstock, 1999, Wan et al., 2008). In a quantitative study, nerve compression for about 8 seconds induced an immediate elevation of the extracellular ATP concentration (Drumev et al., 1983). Additionally, it has been estimated that release of only 1% or less of the intracellular ATP pool is sufficient to activate any and all purinergic receptors (Stoianov and Vulchanova, 1962).

Upon nerve injury, several neurotrophic factors and regeneration-related molecules are induced in DRG neurons and/or non-neuronal cells, and their signalling pathways contribute to the increased growth status as discussed above (Richardson et al., 2009). We suggest that the first step in these processes is likely to be the release from neurons and non-neuronal cells of pre-formed intracellular molecule(s) which acts on non-neuronal cells to release neuropoietic cytokines, and which in turn induce the release and/or synthesis of neurotrophic factors from other non-neuronal cells. ATP fits the role as an initiating factor since it can be released upon nerve injury (Drumev et al., 1983) and can then activate purinergic receptors to release neurotrophic factors and neuropoietic cytokines (Abbracchio and Ceruti, 2006). Our working theory is shown in Figure 1-4.

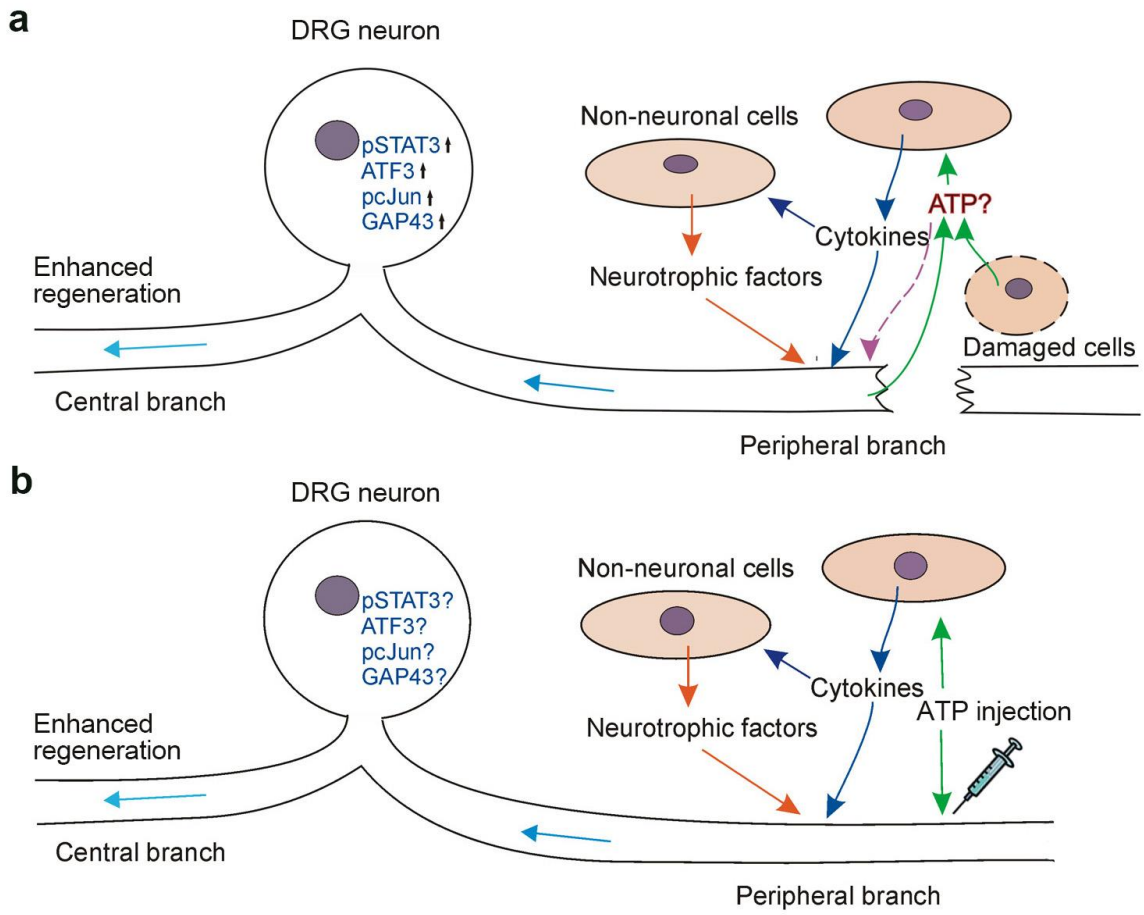


Figure 1-4 Schematic diagrams showing the working theory of ATP. a, Signalling after peripheral nerve injury. **b**, Signalling after ATP injection.

Therefore, our hypothesis is as follows: ATP released upon peripheral nerve injury activates purinergic receptor(s), followed by an increase of neuropoietic cytokines and neurotrophic factors and consequent induction of the expression of growth-related genes, to promote the regeneration capacity of the sensory neurons (Figure 1-4a). We postulate that we can mimic this signalling by injecting ATP into the peripheral (sciatic) nerve (Figure 1-4b)

1.9 Aims of thesis

In this thesis, the overall aim is to mimic a conditioning lesion by injecting ATP into the sciatic nerve to effectively promote axonal regeneration and to better understand the underlying mechanisms involved. As ATP and its metabolites act on purinergic receptors which have a number of different subtypes, it is necessary to define which specific receptor subtype(s) are involved.

Firstly, in Chapter 3, the question of whether injecting ATP into the sciatic nerve could mimic the conditioning lesion effects in vitro (DRG cell culture model) and in vivo (dorsal column transection model) was determined. As it was found effective, the level of neuropoietic cytokines and transcription factors as well as growth associated protein 43 was studied to seek the pathways/mechanisms involved. Morphological changes of the sciatic nerve after injection was also carefully examined.

Although ATP injection into the sciatic nerve could mimic conditioning effects to a certain degree, it was not as effective as a conditioning lesion. Therefore, in Chapter 4, whether a second injection of ATP (one week apart) for the sustained upregulation of transcription factors was more effective than single injection of ATP in promoting axonal regeneration was determined. Furthermore, whether ATP injection affects sensory and motor functions of the sciatic nerve was investigated through behaviour studies.

If the specific receptor subtype(s) which is involved in the ATP-mediated neuroregeneration is identified, it would be useful in the development of more potent agonists or other therapeutic agents. As a first step towards this, the aim of Chapter 5 is to identify all the purinergic receptor subtypes that are present on the sciatic nerve (nerve

fibres and Schwann cells) and DRG (neuronal cell bodies and satellite cells) in mRNA and protein level.

Based on the information obtained from Chapter 5, the remaining future work to be carried out would be to test agonists and antagonists for specific purinergic receptors in an in vitro compartmented culture model using Champenot chambers. After this in vitro experiment, we may use selective purinergic receptor knockout mice to confirm the receptor(s) that are responsible for ATP-mediated growth promoting effects. Completion of this work may lead to the development of potent therapeutic agents to effectively enhance axonal regeneration which someday might be prescribed for spinal cord injured patients in the clinic.

CHAPTER 2 - GENERAL MATERIALS AND METHODS

2.1 Animals

All animal research procedures were performed according to the UK Animals (Scientific Procedures) Act 1986. Adult female Wistar rats weighing 200-250g were used for most of the in vivo experiments. For DRG culture, Wistar rats weighing 100-150 g were used. All rats were supplied by Charles River Ltd. Animals were housed in standard polypropylene cages and maintained under the 12 hours light and 12 hours dark illumination cycle with food pellets and water ad libitum.

2.2 Surgical procedures

2.2.1 Sterilisation of equipment

All surgical instruments, operating gowns, drapes and swabs were autoclaved for 20 minutes at 121°C and then oven dried prior to use.

2.2.2 Anaesthesia

All the animals were initially induced anaesthesia using 4% isoflurane in oxygen delivered from a vaporizer. Once deep anaesthesia was established, i.e. no reflexes could be elicited after toe-pinching, the isoflurane level was reduced to 1.5-2%.

2.2.3 Preparation of the operation

The operating area was shaved using veterinary hair clippers and cleaned with 70% ethanol. Animals were placed on a heated pad and covered with a sterile drape. An anaesthesia nose cone was secured in place.

2.2.4 Dorsal column transection

Surgery was performed using a Zeiss operating microscope. Bilateral T8 thoracic spinal cord dorsal column transection was performed as previously described (Zhang et al., 2007a). An incision was made in the skin at the level of the eighth thoracic vertebra following the direction of the spine. The underlying musculature was incised using a scalpel blade along the bilateral side of the spine to expose the lamina of T8. The T8 spinal cord was exposed by a laminectomy using bone scissors and the dura was opened with fine microsurgical scissors. Subsequently, bilateral dorsal columns were transected with microsurgical scissors at the depth of 1.5 mm from the dorsal surface, and a 29 gauge needle was used to plough the lesion site to assure the complete transection of the dorsal column. Fibrinogen (Sigma) was placed over the transection site in the spinal cord to stop excessive bleeding. The muscle and skin incisions were closed in separate layers with 3/0 sutures. After dorsal column transection, sciatic nerve crush or injections were performed.

2.2.5 Sciatic nerve crush and sham operation control

An incision was made in the skin of the left thigh following the direction of the femur and the underlying quadriceps muscles were separated. The sciatic nerve was exposed at mid-thigh level. The sciatic nerve was crushed for 15 seconds with a pair of #5 jeweller's forceps. The skin incision was closed with 3/0 sutures. Animals with left sciatic nerve exposure without crush were used as a sham operation control.

2.2.6 Sciatic nerve injection

Solutions for the injection were prepared as follows. Sterile 0.9% NaCl (saline) was used. 150 μM ATP were dissolved in saline, respectively. ATP solutions were freshly prepared for each batch of experiments carried out and kept on ice until use. The left sciatic nerve was exposed at mid-thigh level as described above. Injection of 150 μM ATP or saline

(injection control) was performed at mid-thigh level of the left sciatic nerve. All injections of 6 µl per nerve were conducted using a Hamilton syringe with a 31 gauge needle. The needle was inserted into the sciatic nerve to about 1 cm and the needle was slowly withdrawn while injection was performed to spread the injected ATP or saline over a segment of 5 mm in the sciatic nerve. After the injection, the needle was left inside the sciatic nerve for 1 minute to prevent the leakage of the solution through the needle track, and it was then slowly withdrawn. 7-0 sutures were attached at the site of injection for later identification. The muscle and skin incisions were closed in separate layers with 3/0 sutures.

In the case of double injections, the left sciatic nerve was exposed at mid-thigh level as described above. Either 150 µM ATP solution or saline was injected into the left sciatic nerve and 7-0 sutures were attached at the site of injection for later identification. One week later, 150 µM ATP solution or saline were injected into the same previous injected site depending on the group. Double saline injection group was used as an injection control. The surgical procedure of the each group is summarized in Table 2-1. The muscle and skin incisions were closed in separate layers with 3/0 sutures.

Table 2-1 Surgical procedures of the double injection groups

Group	Procedure 1	Procedure 2 (1 week later)
Sham/Sham	Sham operation	Sham operation
Saline/Saline	Saline injection	Saline injection
ATP/Saline	150 µM ATP injection	Saline injection
ATP/ATP	150 µM ATP injection	150 µM ATP injection

2.2.7 Application of the retrograde tracer to the sciatic nerve

Transganglionic tracer cholera toxin subunit B (CTB) was used to label the axons retrogradely (Wan et al., 1982, Trojanowski et al., 1982). Six weeks after the dorsal column transection and the first sciatic nerve injection, the left sciatic nerve was exposed at hip level (above the treatment site) and 5 µl of 1% CTB (List Biological Laboratories Inc, Campbell, USA) was slowly injected into the left sciatic nerve via a Hamilton syringe with 31 gauge needle. The needle was not withdrawn for 1 minute to prevent the leakage of the solution through the needle track. The muscle and skin incisions were closed in separate layers with 3/0 sutures.

2.2.8 Postoperative care

After surgery, 0.02 mg/kg of buprenorphine (Vetergesic, Reckitt Benckiser Healthcare Ltd., Hull, UK) was administered by intraperitoneal injection. All animals were left in an incubator to recover to full consciousness before they were returned to their home cage.

2.3 Perfusion

Animals were overdosed with Sagatal (sodium pentobarbitone, 90 mg/kg; Rhone Merieux, Harlow, Essex, UK) by intraperitoneal injection. When reflexes were no longer present but before respiration completely failed, the ventral abdominal wall was opened along the midline and the diaphragm was cut open. The rib cage was opened with scissors along both sides and pulled back and secured with clamps to expose the heart. A blunt cannula attached to a Watson-Marlow peristaltic pump was immediately inserted into the left ventricle and up into the aorta. The right atrium was opened with a pair of scissors to

release venous blood. The animals were first exsanguinated with 150-200 ml of 0.9% NaCl (saline) and then perfused with 250-300 ml of freshly made 4% paraformaldehyde (PFA) at a flow rate about 20-24 ml/minute. The fixative was made as listed below.

(1) 0.2 M phosphate buffer (PB) (pH 7.4) was prepared from

NaH_2PO_4 x H_2O (monobasic)----------5.5g

Na_2HPO_4 x $12H_2O$ (dibasic)----------57.3g

Distilled H_2O----------1000ml

(2) 80g of paraformaldehyde was dissolved in 1000ml distilled water through heating the solution on a hot plate to a temperature of 70°C while stirring it continuously.

(3) The solution was cleared with a few drops of 10M NaOH.

(4) 1000ml of 0.2M PB (pH 7.4) was added to the solution.

(5) The pH was adjusted to 7.2-7.4 by adding 1M hydrochloric acid (HCl) or 1M NaOH.

(6) The solution was filtered through Whatman No.1 filter paper.

2.4 Dissection

When dissecting tissues for DRG culture all instruments were autoclaved. Animals for immunohistochemistry were perfused, and animals for ELISA, qPCR or DRG culture were not perfused. When dissecting fresh tissues from unperfused animals, animals were anaesthetised with CO_2 and quickly decapitated.

L4-5 DRG were located by tracing the sciatic nerve into the dorsal rami and into the vertebral canal, which was opened via an extensive laminectomy using bone forceps, and they were then removed using fine forceps. The mid-thoracic segments of the spinal cord containing the injury site and upper cervical segments of spinal cord were dissected following a laminectomy. An incision was made in the skin of the left thigh following the direction of femur and the underlying quadriceps muscles were separated. The sciatic nerve was exposed at mid-thigh level. One centimetre around the injection/crush site or the equivalent part of the sciatic nerve (in the case of sham-operated animals) was dissected for ELISA. The entire length of the sciatic nerve was removed in the case of normal animals for qPCR and immunohistochemistry.

2.5 Preparing blocks and sectioning tissues

After perfusion and dissection, all tissues were post-fixed in 4% PFA in 0.1 M PB overnight and then stored in 30% sucrose in 0.1 M PB containing 0.02% sodium azide at 4°C until tissues sank to the bottom of the container. Tissues were removed from the 30% sucrose solution and then placed into plastic embedding wells filled with Shandon M-1 Embedding Matrix (Thermo Fisher Scientific, Altrincham, UK). All blocks were immediately frozen using dry ice and kept at -80°C until sectioning with a cryostat (Leica CM 1900).

Blocks of mid-thoracic spinal cord containing the lesion site (T8 level) were cut in longitudinal orientation at 12 µm; upper cervical spinal cord were cut transversely at 12 µm. L4-5 DRG and sciatic nerve (either longitudinally or transversely) were cut at a thickness

of 8 µm. Sections were mounted onto Superfrost Plus Slides (Fisher Scientific, Pittsburgh, PA, USA) and then air dried overnight before proceeding to be immunostained.

2.6 Immunohistochemistry

2.6.1 Quantification of CTB-labelled axons

For quantification of regenerating axons after the dorsal column transection, animals were perfused 6 weeks after injury/treatments. The mid-thoracic spinal cord segments containing the lesion site and upper cervical spinal cord segments were dissected and sectioned as described above. For identification of CTB-labelled dorsal column axons and lesion cavity, sections were first blocked with blocking buffer (10% normal donkey serum and 1% BSA in TBS); and then double immunostained with goat anti-CTB antibody (1:4000; List Biological Laboratories Inc) and mouse anti-glial fibrillary acidic protein antibody (GFAP, 1:1000; Millipore) in antibody diluting buffer (0.2% Triton X-100 in PBS with 0.1% sodium azide) for 2 days at room temperature; followed by incubation with biotinylated donkey anti-goat IgG antibody (1:400; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG antibody (1:400; Jackson ImmunoResearch Laboratories) in antibody diluting buffer for 2 hours at room temperature. Sections were then further incubated with streptavidin Alexa Fluor 568 antibody (1:2000; Invitrogen) for 2 hours at room temperature. All sections were then mounted with anti-fade reagents containing 2.5% DABCO in PBS/glycerol (1:9).

2.6.2 Detecting transcription factors and GAP43

For immunohistochemistry of transcription factors and GAP43, animals were perfused 3 days after the treatments. L4-5 DRG were dissected and sectioned as described above.

Sections were immersed in 100% methanol at -20°C for 10 minutes, and then in acetic acid at -20°C for 1 minute. After washing in TBS-T (0.05M Tris-HCl, 0.15M NaCl, 0.2% Triton X-100, pH 7.4), tissue sections were blocked in 10% normal goat serum with 1% BSA in TBS-T (blocking buffer) for 1 hour.

Thereafter, sections were incubated with the following primary antibodies diluted in blocking buffer: rabbit anti-ATF3 antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-pcJun antibody (1:200; Cell Signaling Technology, Hertfordshire, UK), rabbit anti-pSTAT3 antibody (1:200; Cell Signaling Technology) or rabbit anti-GAP43 antibody (1:10000; Sigma-Aldrich, Poole, UK) for 2 days at 4°C. Sections were then incubated with Alexa Fluor 568 goat anti-rabbit IgG antibody (1:400 in 3% BSA/TBST; Invitrogen, Paisley, UK) at room temperature for 2 hours. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, 0.5 μg/ml; Sigma-Aldrich) for 10 minutes. Immunostaining of control sections were processed in PBS in place of the primary antibody.

To visualize the subpopulations of DRG neurons, another set of slides were used to stain pSTAT3 or GAP43, together with mouse anti-200-kDa neurofilament (N52 clone, 1:3000, Sigma-Aldrich) for two days at 4°C, followed by incubation with Pacific Blue and mouse anti-AMCA (1:400; Jackson Immunoresearch, Suffolk, UK) for two hours at room temperature, then incubated with biotinylated IB4 (1:200, Sigma-Aldrich) overnight at 4°C and streptavidin Alexa Fluor 488 (1:1000, Invitrogen) for two hours. All sections were then mounted with anti-fade reagents containing 2.5% DABCO in phosphate buffered saline (PBS)/glycerol (1:9).

2.6.3 Morphological change in the sciatic nerve

To examine the morphological change in the sciatic nerve after nerve crushes or ATP injections, animals were perfused 3 days after the treatments. Longitudinal sections (8 μm thick) cut on cryostat were blocked with 10% normal goat serum and 1% BSA in TBS (blocking buffer) for 1 hour. Then, sections were double stained with mouse anti-myelin protein P0 antibody (1:3000; ASTEXX Ltd. & Co. KEG, Graz, Austria) plus rabbit anti-NF200 antibody (1:2000, Sigma-Aldrich), or mouse anti-S100 antibody (1:2000; Sigma-Aldrich) plus rabbit anti-p75 neurotrophin receptor ($p75^{NTR}$) antibody (1:2000, Millipore). All primary antibodies were diluted in blocking buffer and incubated overnight at room temperature. The next day, sections were incubated with Alexa Fluor 568 goat anti-mouse IgG antibody (1:400, Invitrogen) and Alexa Fluor 488 goat anti-rabbit IgG antibody (1:400, Invitrogen) for 2 hours. Control sections were processed in PBS in place of the primary antibody. All sections were then mounted with anti-fade reagents containing 2.5% DABCO in PBS/glycerol (1:9).

2.6.4 Identification of purinergic receptors

To identify the purinergic receptors in DRG and sciatic nerve, normal animals (non-injured, adult female Wistar rats, 200-250g) were perfused, and tissues were dissected and sectioned as described above. Sections were incubated with 10% normal goat serum and 1% BSA in TBS for 1 hour to block non-specific binding. Sections were then incubated with primary antibodies diluted in an antibody diluting buffer (0.2% Triton X-100 in PBS with 0.1% sodium azide): rabbit anti-P2X1 antibody (1:500; Neuromics, Edina, USA), rabbit anti-P2X2 antibody (1:750; Alomone Labs, Jerusalem, Israel), rabbit anti-P2X3 antibody (1:2000; Neuromics), rabbit anti-P2X4 antibody (1:100; Roche, UK), rabbit anti-P2X5 antibody (1:500; Roche), rabbit anti-P2X6 antibody (1:100; Roche), rabbit anti-P2X7 antibody (1:150; Alomone Labs), rabbit anti-P2Y1 antibody (1:100; Alomone Labs), rabbit

anti-P2Y2 antibody (1:50; Alomone Labs), rabbit anti-P2Y4 antibody (1:100; Alomone Labs), rabbit anti-P2Y6 antibody (1:150; Alomone Labs), rabbit anti-P2Y12 antibody (1:50; Alomone Labs), rabbit anti-P2Y13 antibody (1:100; Alomone Labs), rabbit anti-P2Y14 antibody (1:300; Alomone Labs), rabbit anti-A1 antibody (1:300; Alpha Diagnostic International, San Antonio, USA), rabbit anti-A2a antibody (1:30; Santa Cruz Biotechnology, Santa Cruz, USA), or rabbit anti-A2b antibody (1:300; Alomone Labs), rabbit anti-A3 antibody (1:250; Alpha Diagnostic International), as well as guinea pig anti-human protein gene product 9.5 antibody (PGP9.5, 1:250; Neuromics) or mouse anti-S100 antibody (1:1500; Sigma-Aldrich, Poole, UK) for 2 days at room temperature. Control sections were processed in PBS in place of the primary antibody. Then, the slides were incubated with Alexa Fluor 568 goat anti-rabbit IgG antibody (1:1000; Invitrogen), and Alexa Fluor 488 goat anti-guinea pig IgG antibody (1:1000; Invitrogen) or Alexa Fluor 488 goat anti-mouse antibodies, diluted in the antibody diluting buffer and incubated at room temperature for 2 hours. Sections were then mounted with anti-fade reagents containing 2.5% DABCO in PBS/glycerol (1:9).

To examine the specificity of antibodies for purinergic receptors, each antibody was preabsorbed with its antigen peptide (Alomone Labs) at a 4:1 ratio for 24 hours at 4°C. Then, antigen-antibody mixture was centrifuged at 17,000*g* for 30 minutes. The supernatant was pipetted into a clean tube and this preabsorbed serum was used instead of primary antibodies in the above immunohistochemistry process.

Also, sciatic nerves from wild-type and P2X7 receptor knockout mice (kindly provided by GSK) were used to evaluate P2X7 antibody specificity. The tissue was processed as described above and longitudinal sections (8 μm thick) of sciatic nerves were immunostained with rabbit anti-P2X7 receptor polyclonal antibody (1:150; Alomone Labs,

Jerusalem, Israel) for 2 days at room temperature, then, incubated with Alexa Fluor 568 goat anti-rabbit IgG antibody (1:1000; Invitrogen) for 2 hours.

2.7 Image capturing and analysis

2.7.1 Quantification of CTB-labelled axons

For quantification of regenerating axons, CTB-labelled axons in the white matters were counted in 6 – 7 sagittal sections encompassing all CTB-labelled dorsal column axons at 36 μm intervals. For the experiment with single injections (ATP or saline) or sciatic nerve crush, as no axons grew across the lesion cavity, we counted the numbers of CTB-labelled axons at 3 distance points: 0.35 mm, 1.05 mm, 1.75 mm caudal to the border of the lesion cavity (indicated by dashed lines in Fig. 3-2), and those axons growing into the lesion cavity in (sagittal) longitudinal sections. For the double injection experiment, the lesion cavity was divided into three regions: caudal border, lesion centre, and rostral border. In addition, CBT-labelled axons growing into the rostral spinal cord were counted in 3 distance points: 0.35 mm, 1.05mm and 1.75 mm rostral to the border of the lesion cavity.

2.7.2 Detecting transcription factors and GAP43

DRG sections were viewed using a Leica epifluorescence microscope (Leica Microsystems, Wetzlar, Germany) and micrographs were taken with a 20x objective. The percentage of immunopositive neurons for ATF3, pcJun, pSTAT3 or GAP43 over the total numbers of neuronal nuclei (DAPI stained) was calculated by counting 300-500 neurons per each DRG in randomly-chosen sections.

2.7.3 Morphological change in sciatic nerve

Sciatic nerve sections were viewed using a Leica epifluorescence microscope and micrographs were taken were taken at 40x objective in three regions from the sciatic nerve: around the crush or injection site (the central region), 1.4 mm proximal and 1.4 mm distal to the central region. From each animal, four images from each region were taken for quantification. Integrated fluorescence densities of P0, NF200, $p75^{NTR}$ and S100 immunostaining were measured with the ImageJ programme.

2.7.4 Identification of purinergic receptors

Sections were viewed on a Leica epifluorescence microscope, and micrographs were taken either with a 20x (DRG) or 40x (sciatic nerve) objective. The level of expression was determined by naked eyes following the criteria previously described (Xiang et al., 1998).

2.8 Neurite outgrowth assay

Three days after the left sciatic nerve injection or crush, rats were killed by inhalation of a rising concentration of CO_2 and cervical dislocation. The left L4 and L5 DRG were removed and transferred to Hank's solution and digested with 0.125% collagenase (type XI; Sigma-Aldrich) and triturated in 1 ml BSF2 medium [F-12/DMEM, 1× N2 supplement (Invitrogen), 0.3% BSA (Sigma-Aldrich), and 1× penicillin/streptomycin mixture (Invitrogen)] and centrifuged through a cushion of 2 ml 15% BSA at 100*g* for 5 minutes. The cell pellet was resuspended in BSF2 medium and the dissociated neurons were plated at 1,000 cells/well in LAB-TEK II eight well-chamber slides (Nunc, Fisher Scientific UK, Leicestershire, UK) precoated with poly-l-lysine (0.01%; Sigma-Aldrich) and laminin (0.1 μg/well; Invitrogen), and cultured at 37°C with 95% air and 5% CO_2. Sixteen hours later, neurons were fixed with 4% paraformaldehyde and immunostained with a mouse

monoclonal antibody against βIII tubulin (1:500; Sigma-Aldrich), then tetramethylrhodamine isothiocyanate (TRITC)-conjugated donkey anti-mouse IgG (1:400; Jackson Laboratories, USA). The length of the longest neurite of each neuron was measured using ImageJ software. Any neuron with neurites longer than its cell diameter was recorded as a neurite-bearing neuron. Cultures for each group were performed in duplicate, and for each well 200-400 neurons were quantified. Cultures were repeated four times. Neurite length and the percentage of neurite-bearing neurons were analysed.

2.9 Enzyme-linked immunosorbent assay (ELISA)

Three days after the sciatic nerve crush or injections, Left L4-5 DRG and sciatic nerve including injection/crush site (1 cm segment around epicentre of treatment) were taken from deeply anesthetized unperfused rats. Segments of sciatic nerve corresponding to the same positions as those of treated sciatic nerves were also removed from the sham-operated animals. Samples were frozen immediately in liquid nitrogen and kept at -80°C until needed.

2.9.1 Protein extraction

Protein was extracted using PARIS^{TM} Kit (Ambion Ltd., Cambridgeshire, UK) according to the manufacturer's protocol. In brief, tissues were first homogenized in ice-cold Cell Disruption Buffer using a ground-glass homogenizer. Homogenized samples were incubated on ice for 5 minutes and centrifuged at 4°C for 1-2 minutes at top speed in a microcentrifuge. Supernatants were used for protein measurement and ELISA.

2.9.2 Protein measurement

Protein concentrations were measured by DC Protein Assay kit (Bio-Rad, Hertfordshire, UK) and stock solution of BSA (10 mg/ml) was used to prepare a series of protein standards (10, 5, 2.5, 1.25. 0.625, 0.312, 0 mg/ml). Briefly, 5 µl of samples and standards were pipetted into a microplate. 25 µl of Reagent A and 200 µl Reagent B were added into each well. After 15 minutes, absorbance was read at 750 nm with an automate microplate reader (Wallac Victor-1420, Perkin Elmer; Waltham, MA, USA)

2.9.3 ELISA

To detect CNTF in DRG and sciatic nerve (injection/crush site), Rat CNTF DuoSet ELISA kit (R&D Systems, Abingdon, UK) was used. To detect IL-6, Quantikine® Colorimetric Sandwich ELISA kits for rat IL-6 (R&D Systems, Abingdon, UK) were used. Samples and standards were added to the antibody pre-coated microplate. Enzyme Conjugate (Streptavidin conjugated to horseradish-peroxidase), Substrate Solution (tetramethyl benzene) and Stop Solution (1M H_2SO_4) were added one after another according to the manufacturer's protocol. Absorbance was measured at 450 nm with a wavelength correction set to 540 nm using the automate microplate reader (Wallac Victor-1420). Concentrations of CNTF or IL-6 were calculated according to the standard curve of the cytokine and normalized to the protein concentrations.

2.10 Realtime PCR

The normal adult Wistar rats (200-250g) were killed under deep CO_2 anaesthesia. Sciatic nerves and L4-5 DRG were excised, rapidly frozen with liquid nitrogen, and then stored at -80°C until used. Cultured Schwann cells from neonatal Wistar rats (postnatal day 3) were provided by Dr. Juan Luo.

2.10.1 RNA extraction and reverse transcription

Total RNA from each tissue or Schwann cells were extracted using TRIzol® reagent (Invitrogen, Paisley, UK) according to the manufacturer's protocol. Briefly, tissues or cells were homogenized in TRIzol® reagent, followed by a phase separation using chloroform. RNA from the aqueous phase was precipitated by mixing with isopropyl alcohol. After washing the RNA pellet with 75% ethanol, RNA was finally dissolved in RNase-free water. DNA-free™ Kit (Applied Biosystems, Warrington, UK) with recombinant DNase I was used to remove contaminating DNA. First-strand cDNA was synthesized using Superscript® III reverse transcriptase (Invitrogen) and random primers (hexamers, Invitrogen), following the manufacturer's instruction. Briefly, 2 μg total RNA was used in 20 μl reverse transcription reactions containing 1x first strand buffer, 0.5mM each dNTPs, 40U RNaseOUT, 5mM DTT and 200U SuperScript III in the presence of 200 ng random primers.

2.10.2 Primer design

All primers for rat P1 and P2 receptors, apart from P2Y11 which has not been cloned in rats, were designed using Primer 3 and Primer-BLAST program (Table 2-2, custom-synthesized primers from Invitrogen).

Table 2-2 Primer sequences of P2X, P2Y, and adenosine receptors for qPCR

Gene	Primer	Sequence (5' to 3')	Product length
GAPDH	Forward	GCACAGTCAAGGCCGAGAAT	151 bases
	Reverse	GCCTTCTCCATGGTGGTGAA	

P2X1	Forward	AGCATCAGCTTTCCACGCTTCAAGG	239 bases
	Reverse	TTGCAGTGCCGAACGTGCCA	
P2X2	Forward	CCACCACTCGAACTCTCATCAAAGC	227 bases
	Reverse	GGGATGCTTTGGAGTACGCACC	
P2X3	Forward	TTTGGCATCCGCTTTGATGTGCTGG	229 bases
	Reverse	CGAACACTGGGTTGGTTGACGCA	
P2X4	Forward	TGAACCAGACACAGAGCACCTGTCC	212 bases
	Reverse	TAAGAAAGCCGGCGTTGGCACG	
P2X5	Forward	GAGGGGTCAGAAGGAGGATG	211 bases
	Reverse	CTGCTTCACGTTCACAATGG	
P2X6	Forward	GCCACGGCATAAAGACTGGTCAGTG	283 bases
	Reverse	ACAAGGTCCCCGATGCGGAACA	
P2X7	Forward	CCTCAGTGTCCCATCTTCCGGCTAG	279 bases
	Reverse	ACGCACGCCGAAGGCTTTGATC	
P2Y1	Forward	TTCGGGGATGTTATGTGCAAGCTGC	269 bases
	Reverse	TCTGACGTGGAGTCGTAGCAGGTG	
P2Y2	Forward	CTTCAAGTATGTGCTGCTGCCCGTG	247 bases
	Reverse	AAGGAAACGCACCAGCTTGCAGAG	
P2Y4	Forward	TGTATCGACCTTTGCCAGGAGCTGG	296 bases
	Reverse	TTTGCTGCCTCGGCATAGCTGC	
P2Y6	Forward	TACCTAGGCATCTGCCATCC	248 bases
	Reverse	GCTGTGAAGGGAAGCAAGAA	
P2Y11	Not cloned in rats		
P2Y12	Forward	AGGGGTTCAGCCAAAGCTCCCAAG	277 bases

	Reverse	AGCACCTCAGCATGCTCATCAAGGA	
P2Y13	Forward	CACTGGGATGCAGGGCTTCAACAAG	276 bases
	Reverse	CACACAAAACCCCTGAGCTGCCAG	
P2Y14	Forward	AGCCCCTTCTGGTGTCTATCGTCCA	241 bases
	Reverse	AAGACGGTCAGCAGAAGGAACACGA	
A1	Forward	ATTGGGCCACAGACCTACTTC	245 bases
	Reverse	ACCACACTCAGGTTGTTCCAG	
A2a	Forward	CTTCTTCGCCTGTTTTGTCC	223 bases
	Reverse	GTTCCCGTCTTTCTGACTGC	
A2b	Forward	GCTCCATCTTTAGCCTCTTGG	235 bases
	Reverse	ACAGGGCAGCAGCTCTTATTC	
A3	Forward	ACCGATACCTGCGAGTCAAG	211 bases
	Reverse	ATGTAATCCAAGCCGACCAC	

2.10.3 Realtime PCR

Real-time PCR was performed in triplicate with 1 μg cDNA from 3 animals or 3 separate batches of cultured Schwann cells in each tube using a Rotor-Gene 3000 (Corbett Robotics, Sydney, Australia). Each reaction included primers specific for each purinergic receptor subtype or, for calibration, primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a house-keeping gene. Each reaction (10 μl) consisted of cDNA (2 μl), primers (forward and reverse, 0.3 μl each, 0.3 μM final concentration), distilled water (2.4 μl), and QuantiTect™ SYBR® Green PCR Master Mix (Qiagen, Crawley, UK, 5 μl). For PCR, an initial denaturation (95°C, 15 minutes) was followed by 40 cycles of amplification (95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds), with fluorescence measured

at the end of each cycle. The amplification rate was calculated on the basis of a linear regression slope of a dilution row. PCR amplification efficiency (E) was determined based on the equation $E = 10^{[-1/slope]}$ (Higuchi et al., 1993, Rasmussen, 2001). Expression levels of purinergic receptor mRNA relative to GAPDH mRNA level were analyzed from: relative expression = $E^{-Ct(Purinergic)}/E^{-Ct(GAPDH)}$. The authenticity of each real-time PCR product was assessed by melting-curve analysis (65 - 95°C, rising by 1°C at each step, holding for 45 seconds on the 1st step, then, 5 seconds for each subsequent step). All primer pairs gave one specific signal (peak) in melting curves and one band of cDNA of predicted size upon gel electrophoresis (Figure 5-1). Control reactions in which the template cDNA was omitted from the PCR reaction (non-template control, Figure 5-1), or reverse-transcriptase omitted from the reverse transcription reaction (non-RT control, data not shown), did not produce any specific amplification products.

2.11 Behavioural tests

To investigate whether intraneural injection of ATP has any (adverse) effects on sciatic nerve functions, behavioural tests on sensory function (mechanical and thermal thresholds), motor function (footprint analysis) and sensorimotor function (grid walking) were carried out. Wistar rats were divided into 4 groups (5 per group): sham operated control, intraneural injection of saline/saline, ATP/saline and ATP/ATP. The animals were acclimatised to the behavioural equipment for 10 minutes every day for one week prior to the tests. Three baseline readings were taken prior to the treatments (either sham or sciatic nerve injections). Behavioural tests were carried out on day 2 and 6 after the first injection. The second intraneural injection was performed on day 7, and behavioural tests were

continued at day 9, 13, 16, and 20. The experimenter was blind to treatment groups throughout the testing period.

2.11.1 Mechanical sensitivity (Von Frey) test

Mechanical sensitivity was assessed by measuring paw withdrawal threshold using Von Frey hairs according to the 'up-down' method as previously described (Dixon, 1980, Chaplan et al., 1994). Rats were placed in a plastic cubicle with a wire mesh grid bottom which allows full access to the paws and on each testing occasion, behavioural accommodation was allowed for 10 minutes. The test area was the mid-plantar left and right hind paw, in the sciatic nerve distribution, avoiding footpads. The Von Frey hair was applied perpendicular to the plantar surface of the hind paw until it bent and was then held in position for 3 seconds. Each stimulus was presented at intervals of several seconds. A sharp withdrawing of the paw that is not associated with locomotion was considered as a positive response. Eight Von Frey hairs ranging from 0.4 g to 15 g were used. Each test was initiated with application of the 2 g hair filament, in the middle of the series. In the absence of a positive response, stronger hair was applied and in the case of a positive response, the next weaker stimulus was presented, until a change in response was observed. Four additional responses were then assessed sequentially up or down based on the response of the rat. In the case of continuous positive or negative responses until the exhaustion of the hair set, 15 g and 0.4 g were assigned respectively. The 50% paw withdrawal threshold was determined using the formula:

$$50\%\ \text{g threshold} = (10^{[Xf+kd]})$$

Xf = value (in log units) of the final Von Frey hair used

k = tabular value for the pattern of positive/negative responses

d = mean difference (in log units) between stimuli

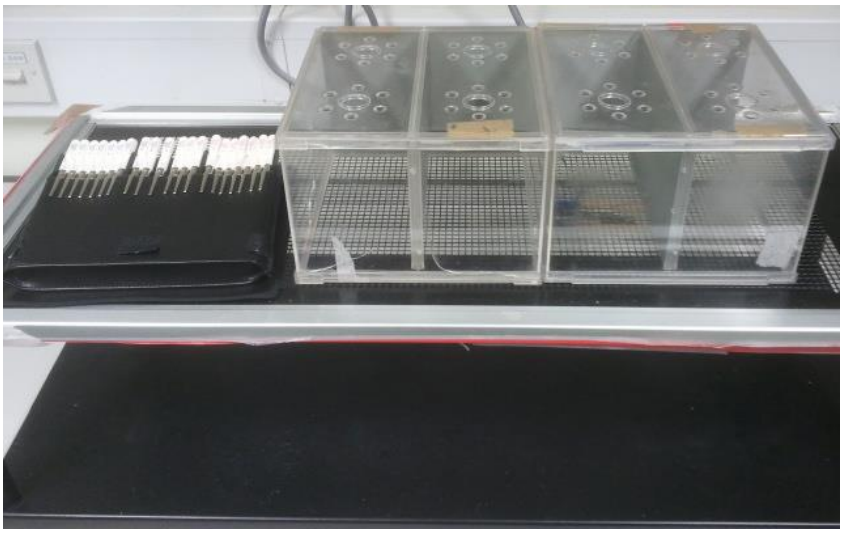


Figure 2-1 Equipment for mechanical sensitivity (Von Frey) test. A Von Frey hair set and plastic cubicles with a wire mesh grid bottom.

2.11.2 Heat sensitivity test

Rats were placed in a plastic cubicle with a transparent glass bottom which allowed full observation of the paw position and access to the paws. On each testing occasion, behavioural acclimatisation was allowed for 10 minutes. Noxious thermal sensitivity was assessed using the Hargreaves' method (Hargreaves et al., 1988), by measuring the time taken for a radiant heat source (143 mV per square cm, 34.1 seconds of maximum reaction time) to elicit a flexion reflex.

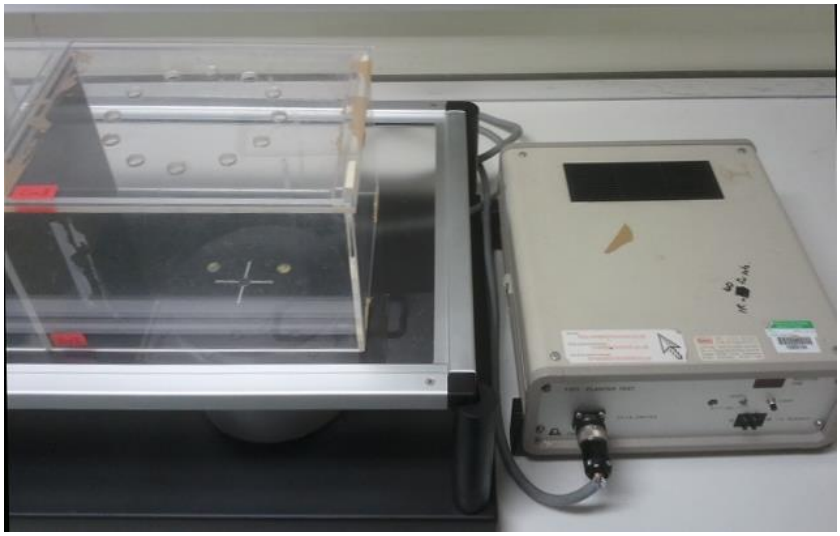


Figure 2-2 Equipment for heat sensitivity test. A radiant heat source and a plastic cubicle with a transparent glass bottom.

2.11.3 Footprint test

For footprint analysis, rat hindpaws were covered with black ink to record walking patterns during continuous locomotion across a wooden runway (7 x 115 cm). The 1st to 5th toe spread of hindpaw on the footprint was measured and used as an early indicator for the recovery of sciatic motor function (Walker et al., 1994, Bain et al., 1989, Bozkurt et al., 2011).

Figure 2-3 Equipment and a drawing showing the measurement of the footprint. a, A wooden runway covered with the recording paper. **b**, TS: the toe spread, distance between the first and the fifth toe.

2.11.4 Grid walking

Rats were trained to cross an elevated metal square grid (40 x 65 cm with 5 x 5 cm grid squares). A video camera was located below the grid apparatus with an angle of about 20–40 degrees. Behaviour on the grid was recorded on video tapes and later hindlimb slips (determined by a paw slipping below the plane of the grid) were counted out of the first 30 steps.

Figure 2-4 Equipment for grid walking test. An elevated metal square grid apparatus.

2.12 Statistics

All statistic tests were performed using IBM SPSS Statistics software. To compare means, two-tailed independent (unpaired) sample t-test as well as one-way or two-way analysis of variance (ANOVA) with post-hoc comparisons and Bonferroni correction were used. The statistical tests used for individual experiments are specified in each figure. $P < 0.05$ was considered to be statistically significant. All data were presented as means ± standard error of the mean (SEM).

CHAPTER 3 - PROMOTING SENSORY AXONAL REGENERATION BY SINGLE ATP INJECTION INTO SCIATIC NERVE

3.1 Abstract

It has been known for several decades that lesion of a peripheral nerve can significantly enhance the regenerative capacity of the corresponding DRG neurons, a phenomenon termed conditioning lesion. However, as a conditioning lesion is a nerve injury, it is not clinically applicable as a therapeutic method for spinal cord injured patients. An alternative method to mimic a conditioning lesion is therefore needed to enhance axonal regeneration. As ATP is released at the injury site following nerve injury and can then release neuropoietic cytokines and neurotrophic factors, we postulated that ATP might play a role in the conditioning lesion. In this chapter, we explored whether ATP injection into a peripheral (sciatic) nerve could mimic the regenerative effects of a conditioning lesion. First, we demonstrated that injection of ATP into the sciatic nerve significantly promoted neurite outgrowth of dissociated DRG neurons in vitro. Furthermore, ATP injection enhanced the regeneration of ascending sensory axons into the lesion cavity after dorsal column transection in vivo; however, single ATP injection was less effective than sciatic nerve crush. Changes in neuropoietic cytokines, transcription factors and growth associated protein were analysed to identify the downstream pathways involved in ATP induced elevation of regenerative state of DRG neurons. ELISA showed that neuropoietic cytokines CNTF and IL-6 levels were significantly increased after ATP injection. In addition, the activated form of transcription factor STAT3 and GAP43 were significantly upregulated in DRG neurons following ATP injection into the sciatic nerve. Since CNTF and IL-6 both signal through the JAK/STAT3 pathway, this pathway is thought to be involved in ATP-mediated conditioning-like effects. Also, morphological analysis of the sciatic nerve showed that ATP injection caused little Wallerian degeneration at the injection site. The results from this chapter demonstrate that ATP injection into the rat sciatic nerve can

partially mimic the conditioning effects, leading to an enhanced regenerative state of the DRG neurons.

3.2 Introduction and aims

Conditioning lesion is a peripheral nerve injury that switches neurons to an active 'regeneration' mode, which can be useful to enhance CNS regeneration in mammals. Although the conditioning lesion has been used frequently in animal models to promote sensory axonal regeneration as a component of combined therapies (Kadoya et al., 2009, Zhang et al., 2007b, Blesch et al., 2012), it is clinically impracticable.

Since injury to nerves caused the release of significant amounts of ATP from injured cells (Drumev et al., 1983) and ATP has been implicated in the release of neuropoietic cytokines and neurotrophic factors (Abbracchio and Ceruti, 2006), we speculate that ATP may play an important role in the mechanism of the conditioning lesion. If so, injection of ATP into peripheral (sciatic) nerves may be able to mimic the effects of the conditioning lesion.

To test whether an ATP injection can mimic the effect of a conditioning lesion, we first designed in vitro (DRG culture to measure neurite outgrowth) and in vivo (dorsal column transection model) experiments to directly measure the degrees of neuroregeneration after ATP injection and compared the results with the effects of sciatic nerve crush (conditioning lesion). Next, we designed the experiments to measure the molecules (cytokines, transcription factors and growth associated protein; CNTF, IL-6, STAT3, GAP43, etc) involved in the conditioning lesion to examine how this ATP-mediated conditioning-like effects is related to the mechanisms of the conditioning lesion

and to what extent it is related. Furthermore, morphological changes of the sciatic nerve after injections were investigated.

Therefore, the aims of this chapter are as follows:

a) To examine whether ATP injection into the sciatic nerve can mimic the growth-promoting effects of the conditioning lesion (in vitro).
b) To examine whether ATP injection into the sciatic nerve can mimic the growth-promoting effects of the conditioning lesion (in vivo).
c) If so, to look for the underlying mechanisms and pathways involved by measuring cytokines, transcription factors and growth associated protein after ATP injection.
d) To examine whether ATP injection cause morphological changes of the sciatic nerve.

3.3 Methods

Briefly, rats were divided into the four treatment groups: sham operation, sciatic nerve crush, saline injection and ATP injection. First, three days after the treatments, DRG were taken and cultured for 16 hours to measure neurite outgrowth. Second, dorsal column transection was performed (sciatic nerve treatments were performed right after the dorsal column transection) and six weeks later, CTB tracer was injected into the sciatic nerve to detect the growing axons in the dorsal column injury (transection) site. Third, three days after the treatments, the sciatic nerve (around injection/crush site) and the corresponding L4-5 DRG were also taken to measure neuropoietic cytokines CNTF and IL-6 using ELISA. Fourth, three days after the treatments, L4-5 DRG were taken to detect transcription factors and growth associated protein changes. Lastly, three days after the treatments, the sciatic

nerve was taken to examine any morphological changes around the crush/injection sites. Details of the experiments can be found in Chapter 2.

3.4 Results

3.4.1 ATP promotes neurite outgrowth in DRG neurons

In this study, we first investigated whether injection of ATP into rat sciatic nerves could promote neurite outgrowth of dissociated DRG neurons. Four groups of adult rats were subjected to sham operation, sciatic nerve crush (conditioning lesion), saline injection, and ATP (150 μM) injection into the sciatic nerve. Three days post treatment, DRG neurons were isolated from the animals and cultured.

After 16 hours in culture, 23 ± 2% of DRG neurons in sham-operated group grew neurites longer than the diameters of their cell bodies (neurite-bearing neurons, Figure 3-1 a, b). The percentage of neurite-bearing cells in the sciatic nerve crush group was significantly increased, to 68 ± 2% ($P = 3\times10^{-8}$, one-way ANOVA test). In the saline group, 31 ± 3% neurons grew neurites longer than the diameters of their cell bodies, which was not significantly different from the sham-operated group. ATP injection increased the neurite-bearing cells to 57 ± 2%, which was significantly higher than in the saline injection group ($P = 5\times10^{-5}$, one-way ANOVA test).

Similar pattern of results were obtained by measuring the longest neurite of each neuron (Figure 3-1 c). Neurons from the nerve crush group had much longer neurites than those from the sham-operated group (147 ± 8 vs. 27 ± 4 μm; $P = 2\times10^{-9}$, one-way ANOVA test). In the saline group, the longest neurite length was 47 ± 7 μm, which was not significantly longer than the sham-operated group, but significantly shorter than the ATP

group (111 ± 4 μm; $P = 2\times10^{-5}$, one-way ANOVA test). There was no significant difference between the ATP injection group and the sciatic nerve crush group in both the percentage of neurite-bearing neurons and the longest neurite length of each neuron. These results show that intraneural ATP injection can elevate the regeneration capacity of DRG neurons in vitro.

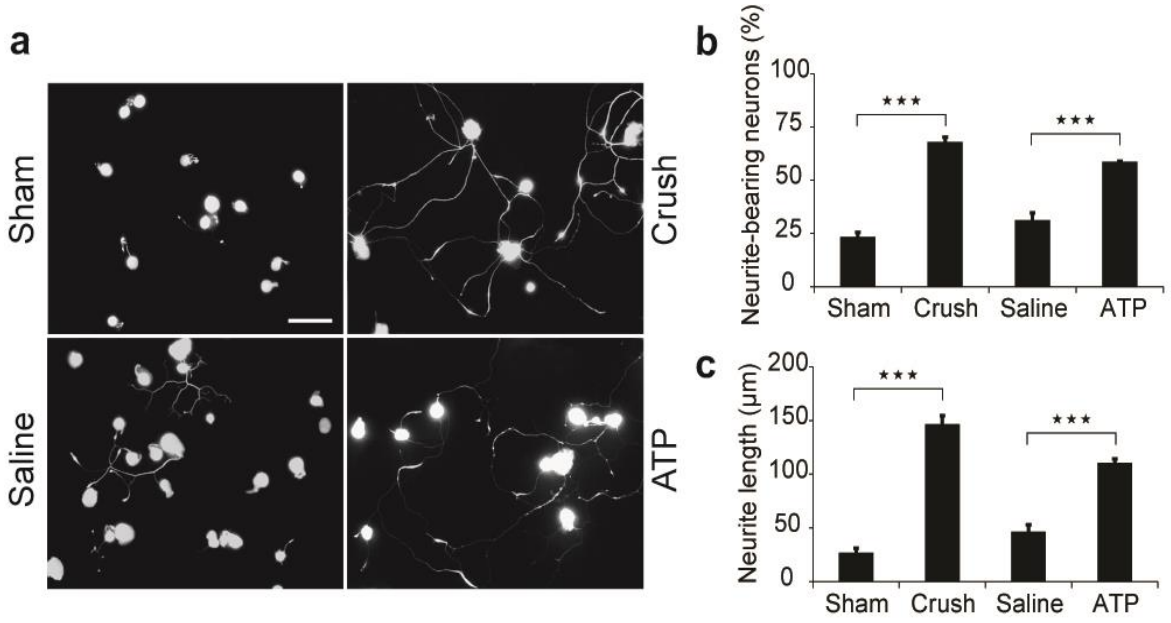


Figure 3-1 Intraneural injection of ATP enhances neurite outgrowth of DRG neurons. **a**, Photomicrographs showing cultured DRG neurons from four experimental groups: sham operation, sciatic nerve crush, sciatic nerve injections of saline or 150 μM ATP. DRG neurons and their neurites are immune-labelled with monoclonal anti-mouse βIII tubulin antibody. Scale bar, 100 μm. **b**, **c**, Quantification of the percentages of neurite-bearing neurons (neurons with neurites longer than their cell bodies; **b**) and the longest neurite lengths of each neuron (**c**) in the four groups. Data are presented as means ± SEM. *** $P <$ 0.001, one-way ANOVA with post-hoc comparisons and Bonferroni correction, n = 4.

3.4.2 ATP enhances axonal regeneration after dorsal column transection

To investigate whether intraneural injection of ATP can promote the regeneration of the ascending sensory axons after spinal cord injury, the T8 spinal cord dorsal column was bilaterally transected in 24 adult rats, which were then divided into four treatment groups as described above. Transganglionic tracer CTB was injected into the sciatic nerve to label the ascending sensory axons in the dorsal column. Six weeks after surgery, the animals were sacrificed and spinal cord segments containing the lesion site were removed, sectioned and stained with an anti-CTB antibody. CTB-labelled axons were seen at various distances caudal to the lesion border in sagittal spinal cord sections in all four groups (Figure 3-2 b). For statistical analysis three distance points caudal to the caudal lesion border (defined by GFAP immunoreactivity; 0.35, 1.05, 1.75 mm) were arbitrarily set up (Figure 3-2 a) and the CTB-labelled axons at each distance point and those inside the lesion cavity were quantified (Figure 3-2 c). More distance points caudal to the caudal lesion border (1.75, 2.45, 3.15 and 3.85mm) were measured to ensure that uptake of CTB injection between groups were similar (data not shown).

At the two points 1.05 and 1.75 mm to the caudal border of the lesion cavity, there was no significant difference in the numbers of CTB-labelled axons among the four groups. However, more CTB-labelled axons grew closer to the lesion border (measured at 0.35 mm caudal to the lesion border) or entered the lesion cavity in the sciatic nerve crush and the ATP injection groups than in the sham-operated and saline injection groups. At 0.35 mm to the caudal lesion border, only 35 ± 3 CTB-labelled axons per animal were counted in the sham-operated group, while significantly more axons (144 ± 21; P = 0.012, one-way

ANOVA test) were counted in the sciatic nerve crush group. In the saline group, 42 ± 8 CTB-labelled axons were counted, which was similar to the sham-operated group, but significantly lower than the ATP group (130 ± 25; $P = 0.027$, one-way ANOVA test). No significant difference in CTB-labelled axon numbers was observed between the sciatic nerve crush and the ATP injection group at 0.35 mm to the caudal lesion border.

Inside the lesion cavity 19 ± 4 and 23 ± 5 CTB-labelled axons were found in the sham-operated and saline groups, respectively (Figure 3-2 c), while significantly more CTB-labelled axons were counted in the sciatic nerve crush group (131 ± 23; $P = 1\times10^{-5}$, one-way ANOVA test) and the ATP injection group (96 ± 16; $P = 0.014$, one-way ANOVA test), respectively. However, the numbers of axons counted in the lesion cavity in the ATP injection group were significantly lower than those counted in the sciatic nerve crush group ($P = 0.049$, one-way ANOVA test). The data from this in vivo study demonstrate that intraneural ATP injection can promote the central sensory axonal regeneration, although the degree of regeneration is less than that following application of the sciatic nerve crush (conditioning lesion).

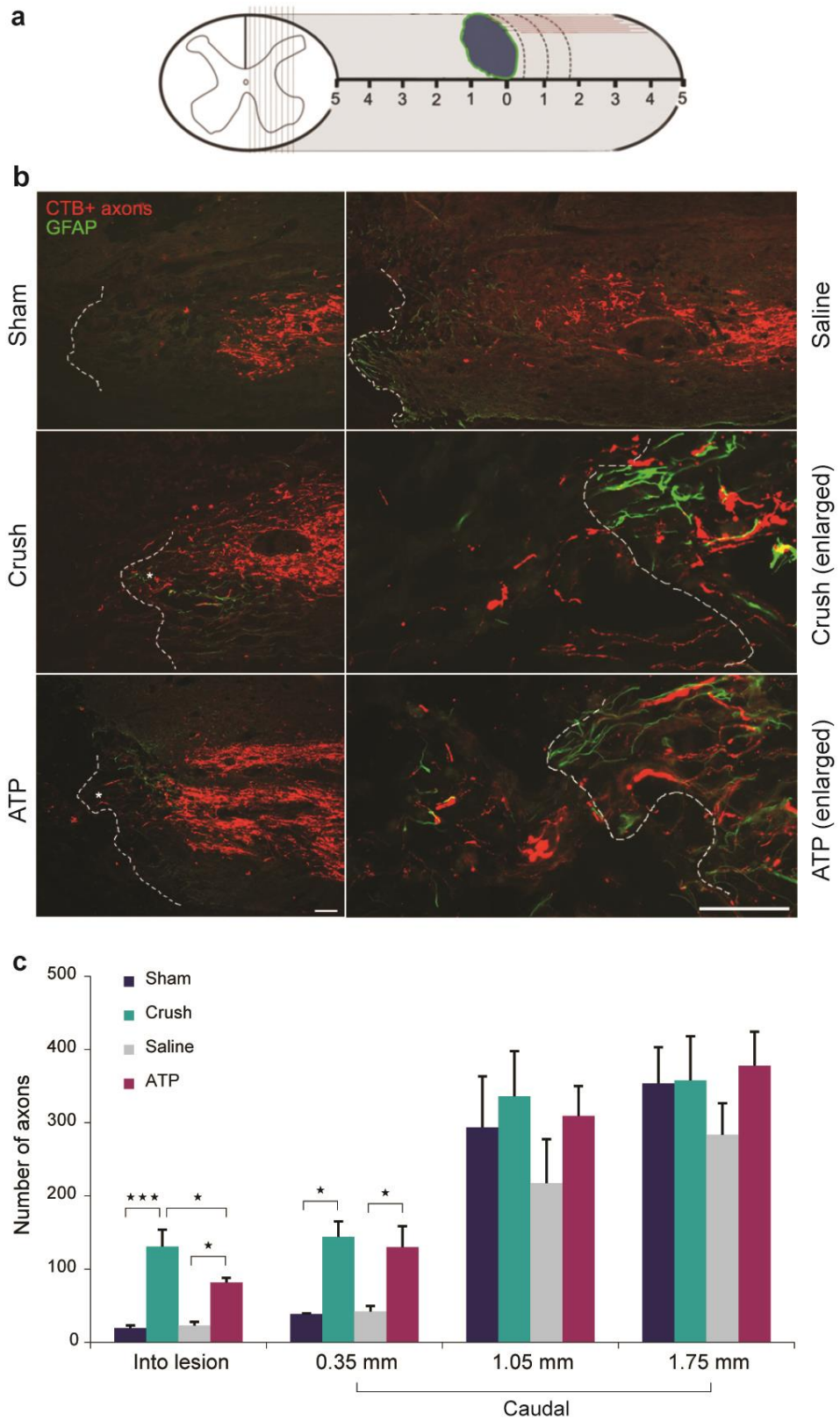


Figure 3-2 Intraneural injection of ATP promotes axonal regeneration after dorsal column transection. a, Schematic drawing showing the segment of thoracic spinal cord

containing the T8 lesion site of dorsal column transection. The ruler shows the distance in millimetres from the border of the lesion cavity. Light grey lines show the sagittal sections through the dorsal column. The green irregular line indicates the glial scar surrounding the lesion cavity. Red lines show CTB-labelled axons. Dotted lines show the 3 distance points to the caudal border of the lesion cavity for counting the axons. **b**, Photomicrographs showing CTB-labelled axons (red) growing towards or beyond the caudal lesion border (defined by GFAP immunostaining, green) in the spinal cord sagittal sections from the 4 groups. To display the CTB-labelled axons at the caudal lesion border and in the lesion cavity more clearly, the regions at the lesion borders in the photomicrographs for the sciatic nerve crush and ATP injection treatments (marked with asterisks) are shown in higher magnification in the panels on the right. Scale bars, 100 μm. **c**, Quantification of CTB-labelled axon numbers in the lesion cavity and at the caudal side of the lesion cavity. Data are presented as means ± SEM. * $P < 0.05$, *** $P < 0.001$, one-way ANOVA with post-hoc comparisons and Bonferroni correction, n = 6.

3.4.3 ATP increases neuropoietic cytokines in sciatic nerve and DRG

Since ATP injections into the sciatic nerve were found to be effective at inducing axonal regeneration in both in vitro and in vivo models, the underlying mechanism for this regenerative effect by ATP was investigated. Neuropoietic cytokines such as CNTF, LIF and IL-6 have been implicated in playing an important role in the increased growth status of axotomised neurons and to be critical in the conditioning lesion (Cafferty et al., 2004, Cao et al., 2006, Cafferty et al., 2001, Richardson et al., 2009). Therefore, to examine

whether neuropoietic cytokines are involved, we measured the levels of CNTF and IL-6 at the injection (or crush) site in sciatic nerve and in L4-5 DRG three days after the treatments.

There was an upregulation of the CNTF protein level in both DRG and the sciatic nerve after sciatic nerve crush or intraneural injection of ATP, detected using ELISA (Figure 3-3). Sciatic nerve crush significantly increased CNTF in both DRG ($P = 0.002$, one-way ANOVA test) and the sciatic nerve ($P = 3\times10^{-5}$, one-way ANOVA test), compared with sham-operated animals. ATP injection also significantly increased CNTF in both DRG ($P = 1\times10^{-7}$, one-way ANOVA test) and the sciatic nerve ($P = 0.002$, one-way ANOVA test), compared with the injection of saline. Interestingly, the level of CNTF was significantly higher in the ATP injection group, compared with the sciatic nerve crush group, in both DRG ($P = 1\times10^{-7}$, one-way ANOVA test) and the sciatic nerve ($P = 1\times10^{-6}$, one-way ANOVA test). These data suggest that ATP injection into the sciatic nerve increases CNTF levels in DRG and the sciatic nerve.

In the sciatic nerve crush group, IL-6 protein levels were significantly higher than those in the sham-operated group in both DRG ($P = 6\times10^{-5}$, one-way ANOVA test) and the sciatic nerve ($P = 1\times10^{-6}$, one-way ANOVA test), detected using ELISA (Figure 3-4). ATP injection also significantly increased the IL-6 levels in both DRG ($P = 2\times10^{-4}$, one-way ANOVA test) and the sciatic nerve ($P = 0.005$, one-way ANOVA test), compared with the saline injection group. The IL-6 level in DRG from the ATP injection group was similar to that of the sciatic nerve crush group. However, the IL-6 level of ATP group was significantly lower than the sciatic nerve crush group in the sciatic nerve ($P = 6\times10^{-5}$, one-way ANOVA test). These data suggest that ATP injection into the sciatic nerve increases IL-6 levels in DRG and the sciatic nerve. Furthermore, these results also demonstrate that

ATP injection into the sciatic nerve can mimic the conditioning lesion by inducing neuropoietic cytokines CNTF and IL-6.

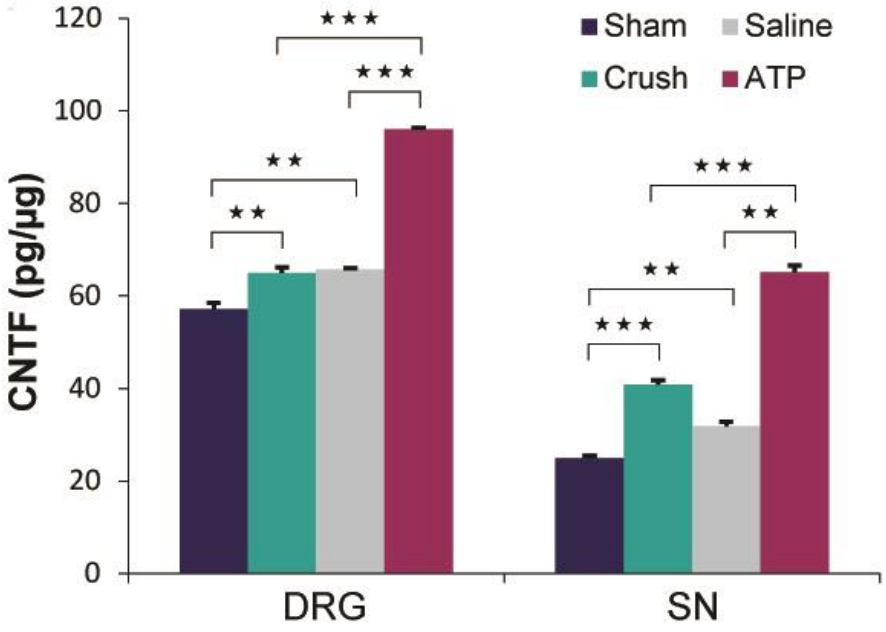


Figure 3-3 Intraneural injection of ATP increases the levels of CNTF in DRG and sciatic nerve. Sandwiched ELISA was used to measure the levels of CNTF in protein extracts from the treated segments of sciatic nerve and L4-5 DRG from the rats subjected to either sciatic nerve crush, injections of saline or 150 µM ATP. DRG and sciatic nerve tissues were taken three days after treatments. The segments of sciatic nerve corresponding to those segments of the treated nerves were taken from the sham operated rats and used as a control. Sciatic nerve crush and ATP injection induced a significant increase in CNTF levels in both DRG and the sciatic nerve. Data are presented as means ± SEM. ** $P < 0.01$; *** $P < 0.001$, one-way ANOVA with post-hoc comparisons and Bonferroni correction, n = 3.

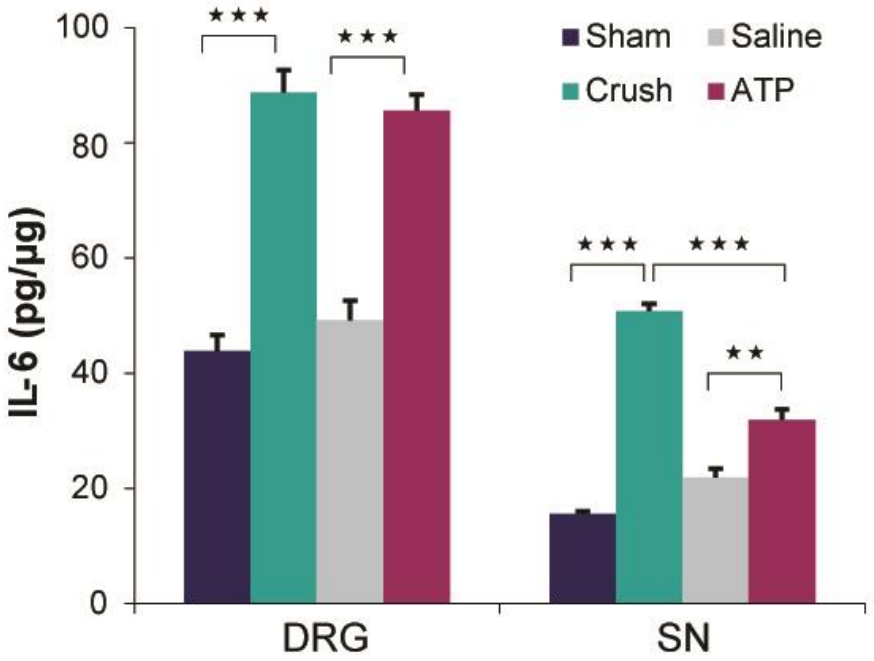


Figure 3-4 Intraneural injection of ATP increases the levels of IL-6 in DRG and sciatic nerve. Sandwiched ELISA was used to measure the levels of IL-6 in protein extracts from the treated segments of the sciatic nerve and L4-5 DRG from the rats subjected to either sciatic nerve crush, injections of saline or 150 µM ATP. DRG and sciatic nerve were taken three days after treatments. The segments of sciatic nerve corresponding to those segments of the treated nerves were taken from the sham operated rats and used as a control. Sciatic nerve crush and ATP injection induced a significant increase in IL-6 levels in both DRG and the sciatic nerve. Data are presented as means ± SEM. ** $P < 0.01$; *** $P < 0.001$, one-way ANOVA with post-hoc comparisons and Bonferroni correction, n = 3.

3.4.4 ATP activates STAT3 signalling in DRG neurons

The neuropoietic cytokines tested above induce the phosphorylation and nuclear translocation of STAT3 (Lee et al., 2004, Qiu et al., 2005). Phosphorylated STAT3 (pSTAT3) then activates the transcription of various genes that support the survival and enhance the growth ability of injured neurons.

In this study, in DRG from sham-operated animals, only 4 ± 1% neurons were $pSTAT3^+$, while sciatic nerve crush significantly activated STAT3 in 36 ± 4% neurons ($P = 1\times10^{-6}$, one-way ANOVA test; Figure 3-5). Although saline injection caused an increase in number of $pSTAT3^+$ neurons (10 ± 1%), this was not significantly different from the sham-operated animals. ATP injection led to an increase of $pSTAT3^+$ neurons to 21 ± 2%, which was significantly higher than the saline group ($P = 0.011$, one-way ANOVA test), but lower than the sciatic nerve crush group ($P = 0.003$, one-way ANOVA test). The results indicate that the STAT3 signalling pathway is involved in an ATP induced increase in the regenerative state of DRG neurons.

Other transcription factors such as ATF3 and cJun are also activated in DRG neurons following a conditioning lesion (Tsujino et al., 2000, Broude et al., 1997, Murray-Rust et al., 2001). Therefore, in this study we examined whether ATF3 and cJun are upregulated after ATP injection.

In DRG from sham-operated animals, only 1 ± 0% neurons were $ATF3^+$, while sciatic nerve crush significantly activated ATF3 in 61 ± 2% neurons ($P = 4\times10^{-8}$, one-way ANOVA test; Figure 3-6). Saline injection also caused a slight increase of $ATF3^+$ neurons (16 ± 2%), which was significantly higher than the sham-operated animals ($P = 0.031$, one-way ANOVA test). ATP injection led to an increase of $ATF3^+$ neurons to 16 ± 5%, which

was also significantly higher than the sham-operated group (P = 0.028, one-way ANOVA test), but similar level to the saline injection group.

The results of pcJun were similar to those of ATF3. Only 2 ± 1% neurons were $pcJun^+$ in a sham-operated group, while sciatic nerve crush significantly increased pcJun in 45 ± 3% neurons ($P = 4\times10^{-8}$, one-way ANOVA test; Figure 3-7). Saline injection also caused a moderate increase of $pcJun^+$ neurons (15 ± 1%), which was significantly higher than the sham-operated animals (P = 0.009, one-way ANOVA test). ATP injection led to an increase of $pcJun^+$ neurons to 11 ± 2%, but it was not significantly higher than that of the saline injection group.

The results suggest that the ATF3 and pcJun signalling pathway may not be involved in the single ATP injection mediated increase of the regenerative state of DRG neurons. There was no difference between the saline and ATP injection groups, indicating the increased expression of ATF3 and pcJun was caused by the injection procedure.

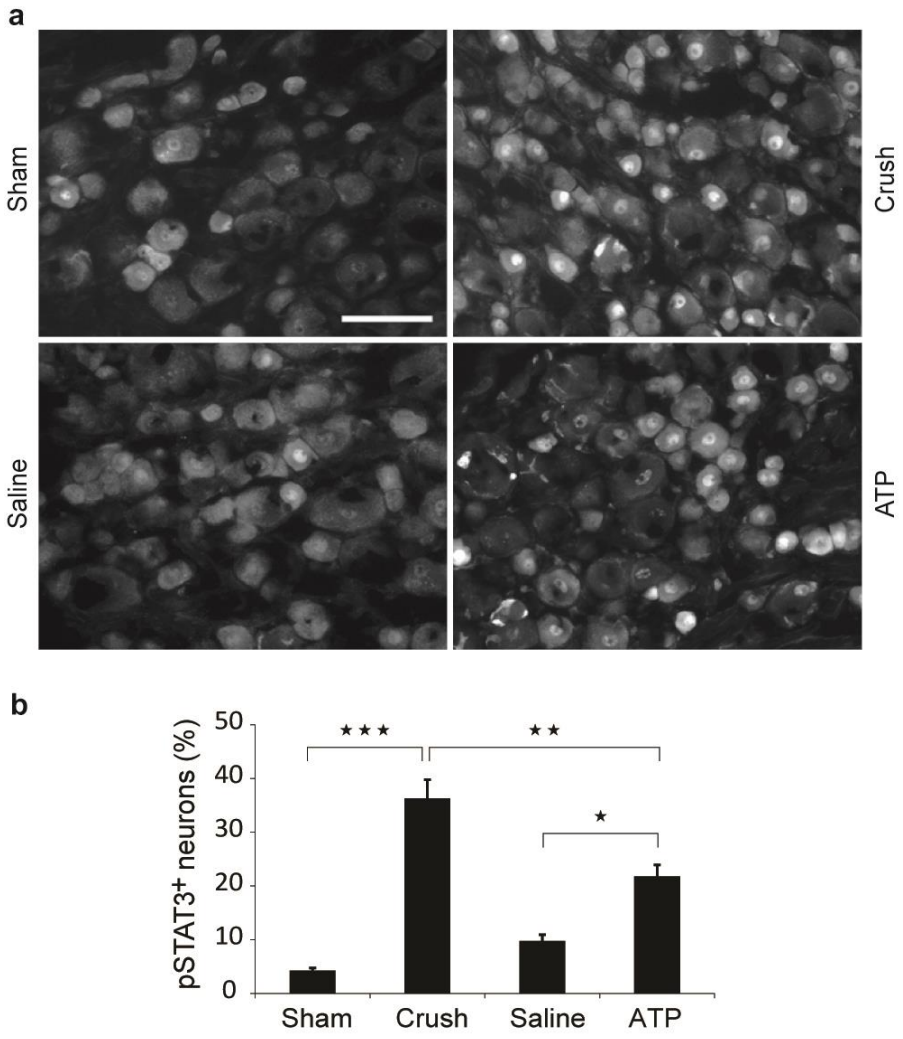


Figure 3-5 Intraneural injection of ATP activates STAT3 in DRG neurons. a, Immunoreactivity for phosphorylated STAT3 (pSTAT3) in L4–5 DRG neurons from four groups: sham operation, sciatic nerve crush, injections of saline or 150 μM ATP into the sciatic nerve. DRG were removed three days after the treatments for immunohistochemistry. Scale bar, 100 μm. **b**, Quantification of neurons with nuclear profiles containing pSTAT3 immunoreactivity. The number of neurons with nuclei containing pSTAT3 immunoreactivity is expressed as a percentage of the total number of neurons with visible nuclei (stained by DAPI). Data are presented as means ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with post-hoc comparisons and Bonferroni, n = 4.

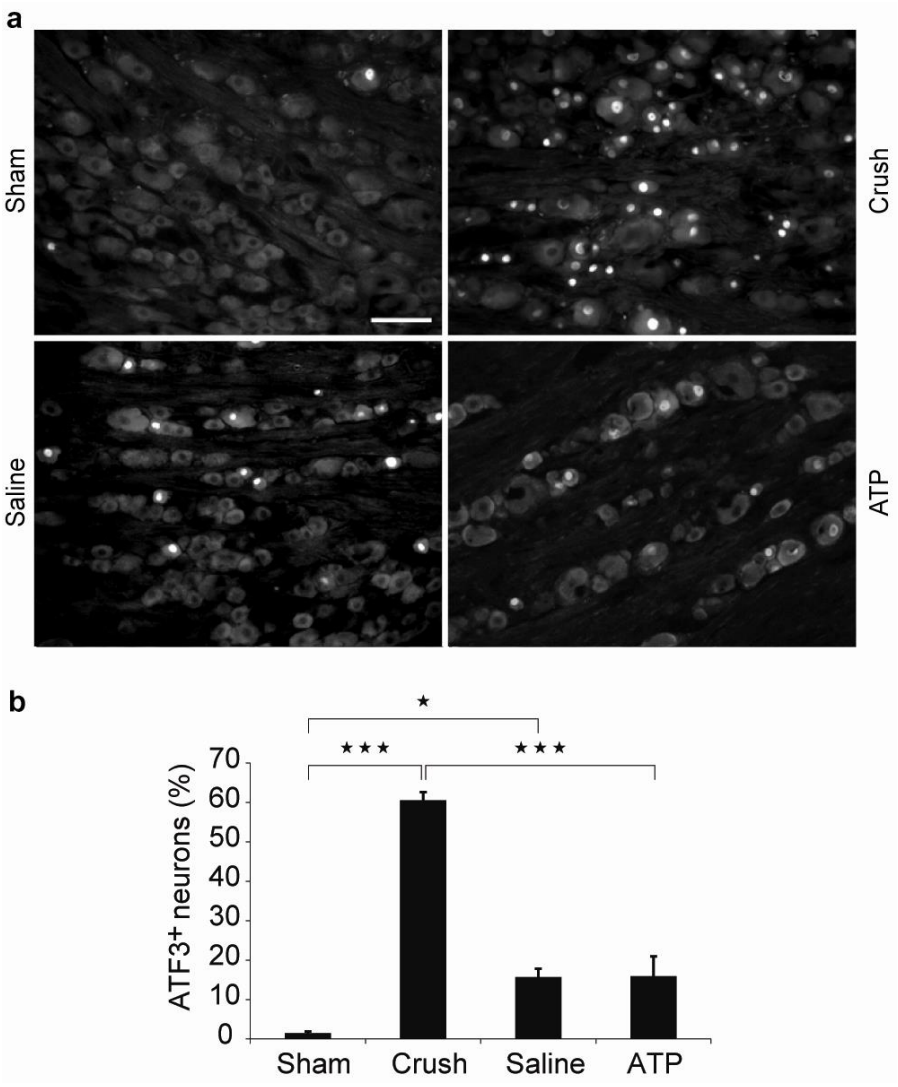


Figure 3-6 Intraneural injection of ATP does not increase ATF3 expression in DRG neurons. a, Immunoreactivity for ATF3 in the nuclei of L4–5 DRG neurons from four groups: sham operation, sciatic nerve crush, injections of saline or 150 μM ATP into sciatic nerve. DRG were removed three days after the treatments for immunohistochemistry. Scale bar, 100 μm. **b**, Quantification of nuclear profiles containing ATF3 immunoreactivity in the nuclei of L4–5 DRG neurons. The number of neurons with nuclei containing ATF3 immunoreactivity was expressed as a percentage of the total number of neurons with visible nuclei (stained by DAPI). Data are presented as means ± SEM. * $P < 0.05$, *** $P < 0.001$, one-way ANOVA with post-hoc comparisons and Bonferroni correction, n = 4.

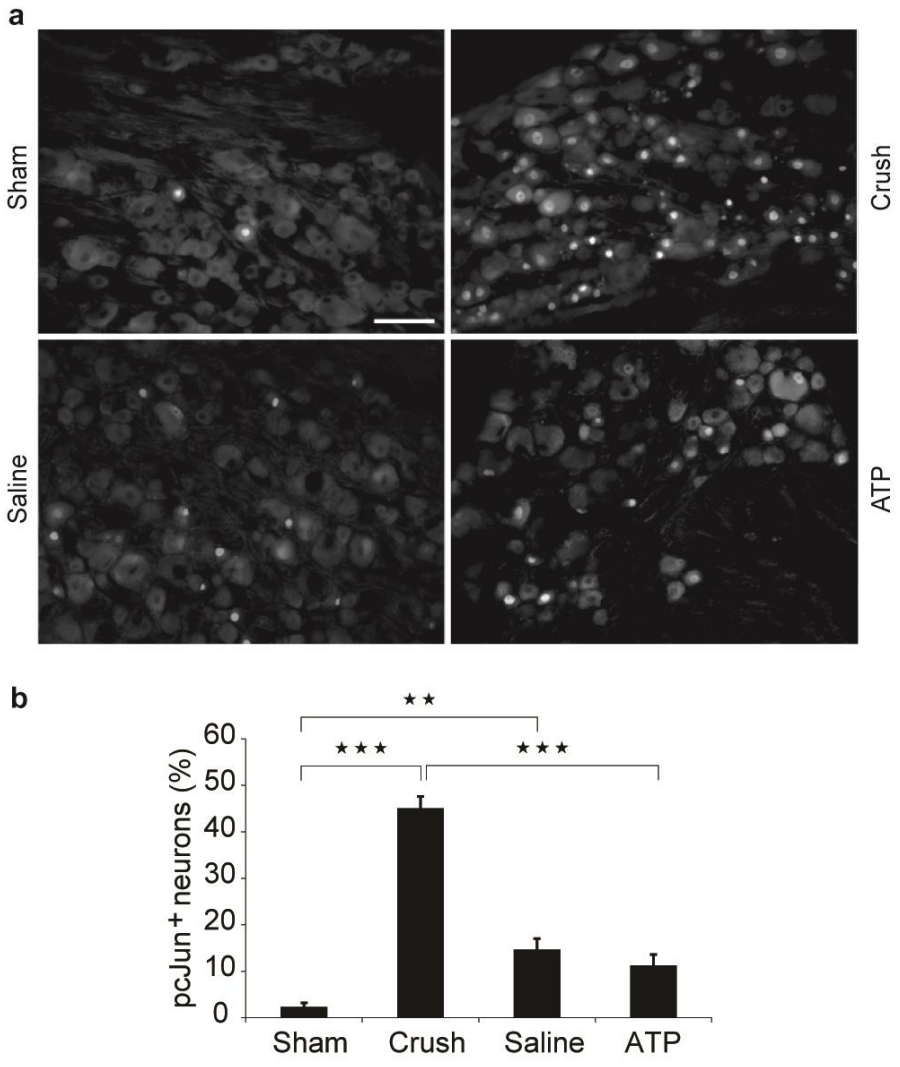


Figure 3-7 Intraneural injection of ATP does not increase cJun activation in DRG neurons. a, Immunoreactivity for pcJun in the nuclei of L4–5 DRG neurons from four groups: sham operation, sciatic nerve crush, injections of saline or 150 μM ATP into sciatic nerve. DRG were removed three days after the treatments for immunohistochemistry. Scale bar, 100 μm. **b**, Quantification of nuclear profiles containing pcJun immunoreactivity in the nuclei of L4–5 DRG neurons. The number of neurons with nuclei containing pcJun immunoreactivity was expressed as a percentage of the total number of neurons with visible nuclei (stained by DAPI). Data are presented as means ± SEM. ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with post-hoc comparisons and Bonferroni correction, n = 4.

3.4.5 ATP increases GAP43 expression in DRG neurons

The expression of GAP43 is considered as a marker for axonal regeneration (Schreyer and Skene, 1993, Skene, 1989), which is increased in DRG neurons following a conditioning lesion (Mulloy and Linhardt, 2001, Hu-Tsai et al., 1994). In this study, GAP43 immunoreactvity were observed in 19 ± 2% DRG neurons of sham-operated animals (Figure 3-8). Sciatic nerve crush significantly increased the number of $GAP43^+$ neurons to 49 ± 3% ($P = 2\times10^{-6}$, one-way ANOVA test). In the saline injection group, 22 ± 1% neurons were $GAP43^+$, which was not significantly higher than the sham-operated animals. ATP injection increased $GAP43^+$ neurons to 35 ± 2%, which was significantly higher than the saline group ($P = 0.004$, one-way ANOVA test), but lower than the sciatic nerve crush group ($P = 0.005$, one-way ANOVA test). The result also shows that ATP injection can enhance the regenerative state of DRG neurons, but it is not as potent as sciatic nerve crush (i.e. a conditioning lesion).

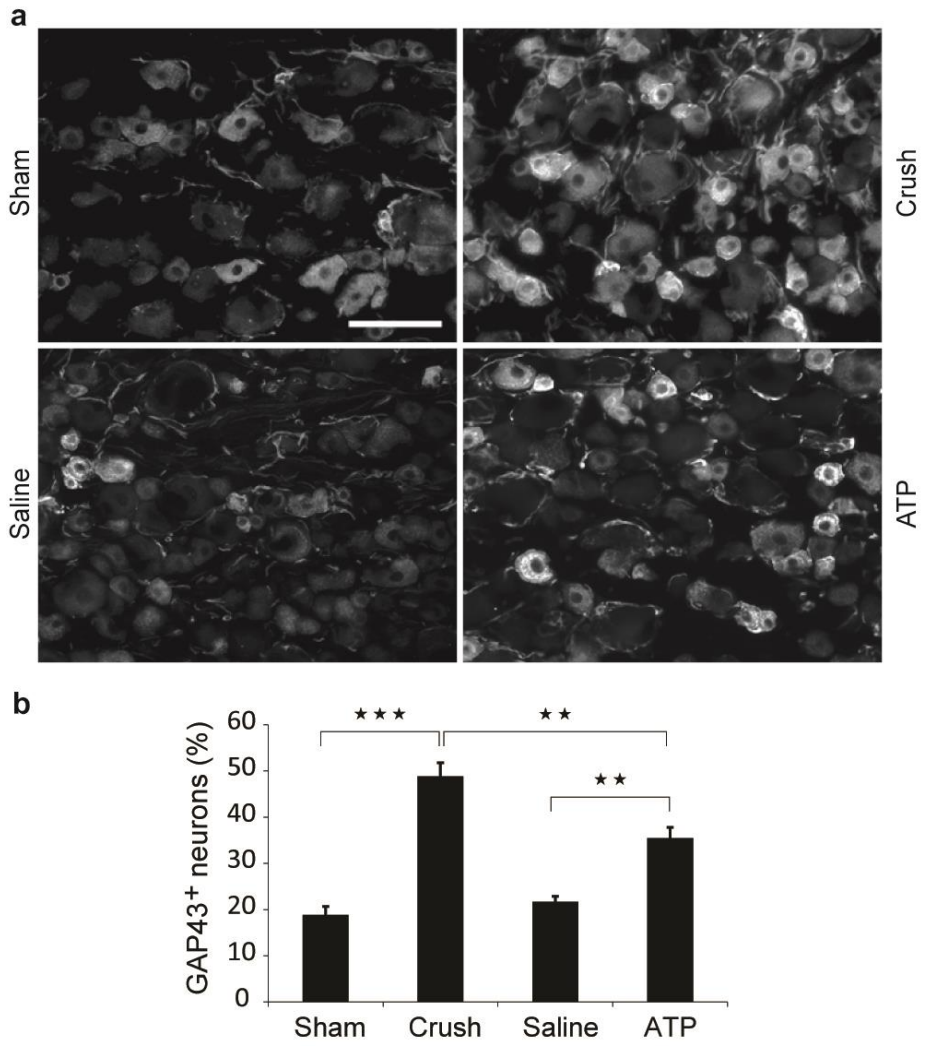


Figure 3-8 Intraneural injection of ATP increases the expression of GAP43 in DRG neurons. a, Immunoreactivity for GAP43 in L4–5 DRG neurons from four groups: sham operation, sciatic nerve crush, injections of saline or 150 μM ATP into sciatic nerve. DRG were removed three days after the treatments for immunohistochemistry. Scale bar, 100 μm. **b**, Quantification of $GAP43^+$ DRG neurons. The number of neurons with GAP43 immunoreactivity in the cytoplasm is expressed as a percentage of the total number of neurons with visible nuclei (stained by DAPI). Data are presented as means ± SEM. ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with post-hoc comparisons and Bonferroni correction, n = 4.

3.4.6 Subpopulation of DRG neurons with activated STAT3 and GAP43 after ATP injection

To identify the subpopulations of DRG neurons with pSTAT3 or GAP43, we triple-labelled DRG neurons for pSTAT3 or GAP43, together with N52 and IB4 binding sites. N52 immunoreactive neurons correspond to large neurons with afferent myelinated (Aβ-fibre) axons innervating sensitive mechanoreceptors peripherally (and therefore subserve tactile and proprioceptive functions) and forming the fasciculus gracilis in the dorsal column (Matsumoto et al., 2007, Bradbury et al., 2000). IB4 immunoreactive neurons are those with unmyelinated (C-fibre) axons innervating nociceptors peripherally and terminating in superficial laminae of the dorsal horn (Matsumoto et al., 2007, Bradbury et al., 2000).

Analysis of the expression of pSTAT3 in $N52^+$ and $IB4^+$ subpopulations of neurons showed that $pSTAT3^+$ neurons were seen in both $N52^+$ and $IB4^+$ subpopulations and a significantly higher percentage of $N52^+$ neurons expressed $pSTAT3^+$ than $IB4^+$ neurons in all four experimental groups: sham-operated (P = 0.049, Student's *t*-test), sciatic nerve crush (P = 0.002, Student's *t*-test), saline (P = 0.047, Student's *t*-test) and ATP injection groups (P = 0.007, Student's *t*-test (Figure 3-9).

Analysis of the expression of GAP43 in $N52^+$ and $IB4^+$ subpopulations of neurons showed similar results to those of pSTAT3. $GAP43^+$ neurons were seen in both $N52^+$ and $IB4^+$ subpopulations and significantly higher percentage of $N52^+$ neurons expressed $GAP43^+$ than $IB4^+$ neurons in all four experimental groups: sham-operated (P = 0.006, Student's *t*-test), sciatic nerve crush (P = 0.002, Student's *t*-test), saline (P = 0.019, Student's *t*-test) and ATP injection groups (P = 0.019, Student's *t*-test) (Figure 3-10). These

data suggest that ATP injection induced STAT3 activation and GAP43 expression in both $N52^+$ neurons and $IB4^+$ neurons.

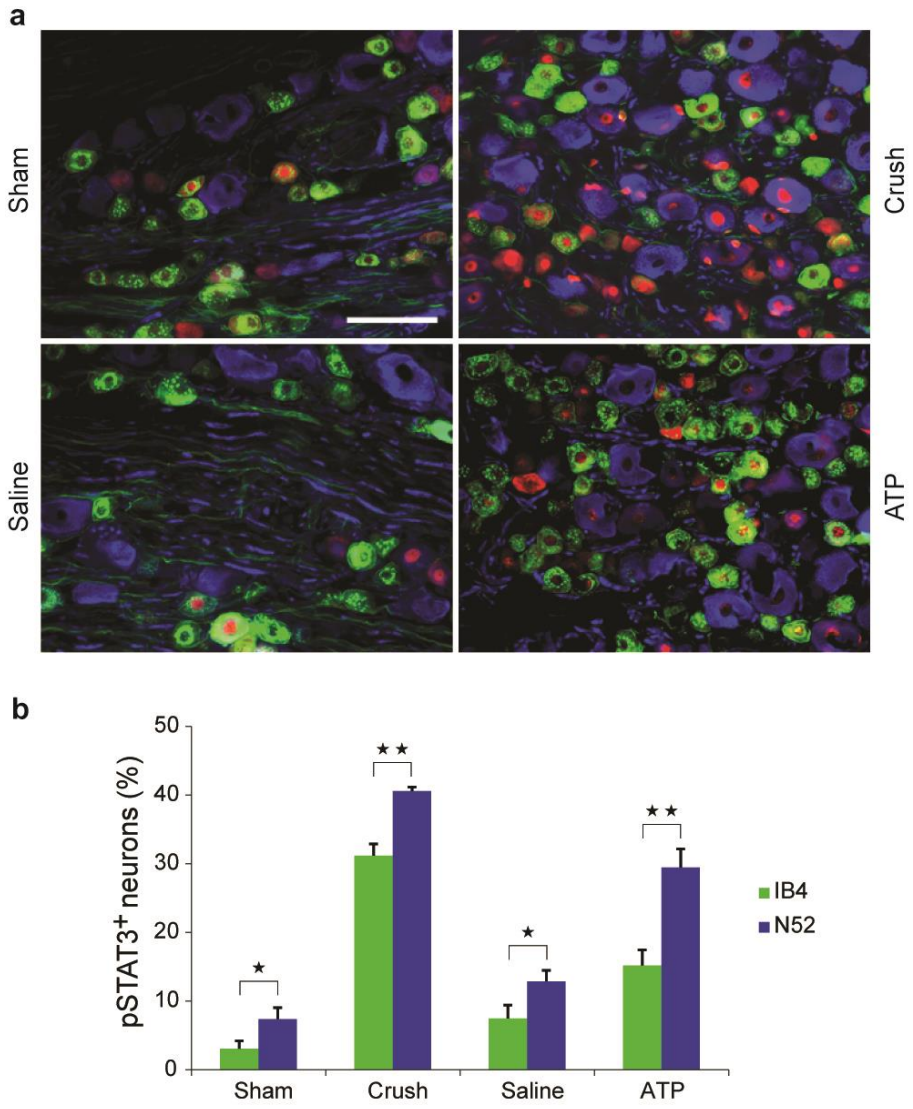


Figure 3-9 Intraneural injection of ATP activates STAT3 in subpopulations of DRG neurons. a, Triple-staining for pSTAT3 (red), N52 (blue), and IB4 binding sites (green) in rat L4–5 DRG from the four groups of rats subjected to after sham operation, sciatic nerve crush, injections of saline or 150 μM ATP into sciatic nerves. Scale bar, 100 μm. **b**,

Percentages of pSTAT3 immunopositive neurons in either the $IB4^+$ or $N52^+$ subpopulation of neurons. Scale bar = 100 μm. * $P < 0.05$; ** $P < 0.01$, Student's *t*-test, n = 4.

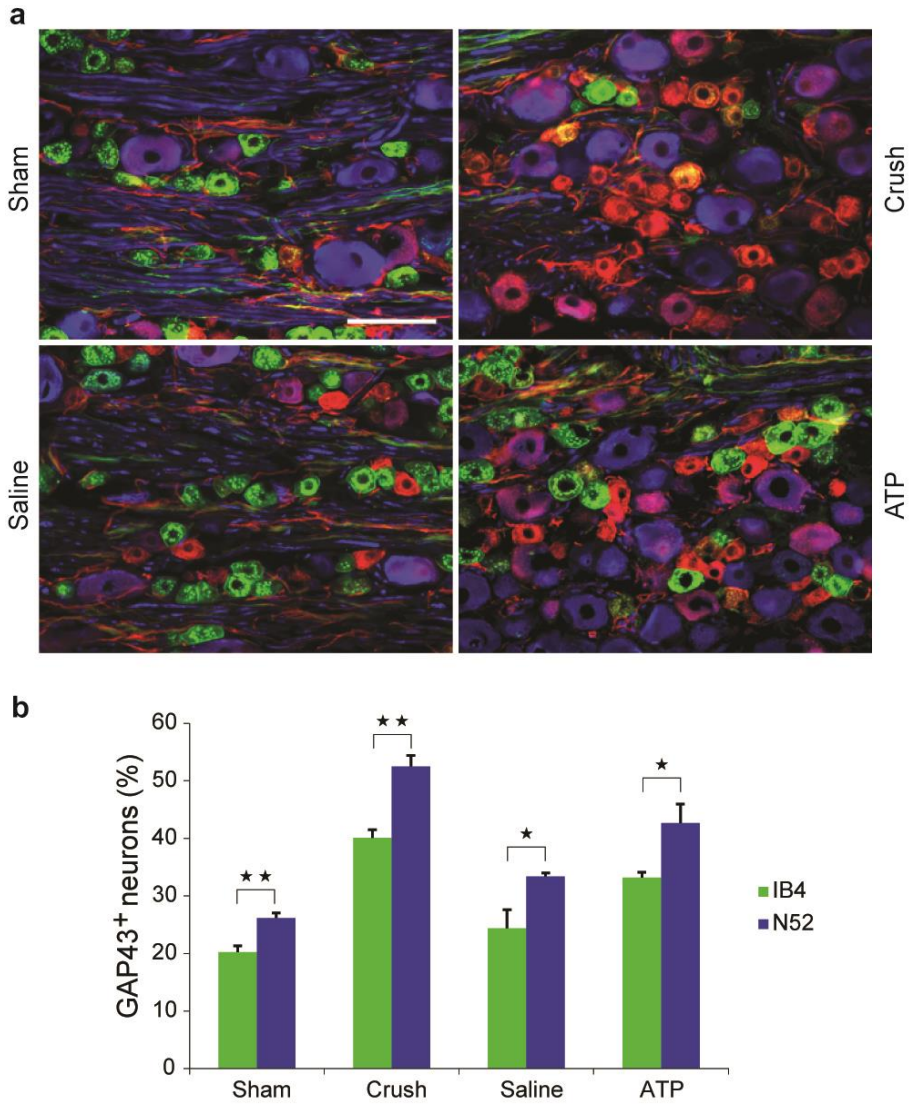


Figure 3-10 Intraneural injection of ATP induce GAP43 expression in subpopulation of DRG neurons. a, Triple-staining for GAP43 (red), N52 (blue), and IB4 binding sites (green) in rat L4–5 DRG from the four groups of rats subjected to after sham operation, sciatic nerve crush, injections of saline or 150 μM ATP into sciatic nerves. Neurons appear yellow in the photomicrographs if they are positive for both GAP43 and IB4, and appear magenta if positive for both GAP43 and N52. Note that neurons with weaker GAP43

immunoreactivity may appear green or blue rather than yellow or magenta in the merged multichannel photomicrographs. Scale bar, 100 μm. **b**, Percentages of GAP43 immunopositive neurons in either the $IB4^+$ or $N52^+$ subpopulation of neurons. * $P < 0.05$; ** $P < 0.01$, Student's *t*-test, n = 4.

3.4.7 Morphological changes in the sciatic nerve after ATP injection

Previously, we have examined the protein changes that occur in the DRG neurons and sciatic nerve after ATP injection. However, it is important to identify any morphological changes that occur at the injection site after ATP injection. Sciatic nerve sections were immunostained for S100 (Schwann cells marker protein), P0 (peripheral myelin protein 0), NF (neurofilament 200), and $p75^{NTR}$ (a marker for de-differentiated Schwann cells during Wallerian degeneration) three days after treatments (sham operation, sciatic nerve crush, injections of saline or 150 μM ATP into sciatic nerve).

As expected, sciatic nerve crush caused substantial tissue damage at the crush site, while intraneural injection of saline and ATP did not cause obvious morphological changes at the injection site (Figure 3-11). First of all, sciatic nerve crush significantly abolished myelin protein P0 immunoreactivity in the crush centre ($P = 1\times10^{-6}$, one-way ANOVA test) and distal region ($P = 0.027$, one-way ANOVA test) of the sciatic nerve, while saline or ATP injection did not significantly affect the P0 immunoreactivity in all three regions (proximal, central and distal) of the sciatic nerve. Similarly, sciatic nerve crush significantly reduced Schwann cell marker S100 immunoreactivity in the crush centre of the sciatic nerve ($P = 0.01$, one-way ANOVA test), while saline or ATP injection did not

significantly affect the S100 immunoreactivity in all three regions (proximal, central and distal) of the sciatic nerve.

The density of neurofilament was significantly reduced in the crushed nerve in central (P = 0.018, one-way ANOVA test) and distal regions (P = 0.045, one-way ANOVA test), and the photomicrograph (Figure 3-11 a, green) shows that the crushed axons were completely disorganized at the crush centre of the sciatic nerve. In the saline and ATP injected sciatic nerve, NF immunoreactivity appears reduced, although not statistically significantly when compared with the sham-operated group, and the axons also maintained their original orderly arrangement. Sciatic nerve crush also significantly increased the expression of the $p75^{NTR}$ in the crush centre (P = 0.002, one-way ANOVA test) as well as in the proximal (P = 0.006, one-way ANOVA test) and distal ($P = 1\times10^{-4}$, one-way ANOVA test) regions to the crush site. Saline and ATP injections also increased the expression of the $p75^{NTR}$ in central and distal regions of the sciatic nerve, although to lesser degrees than sciatic nerve crush, indicating there was mild injury to the sciatic nerve due to the injection procedure itself.

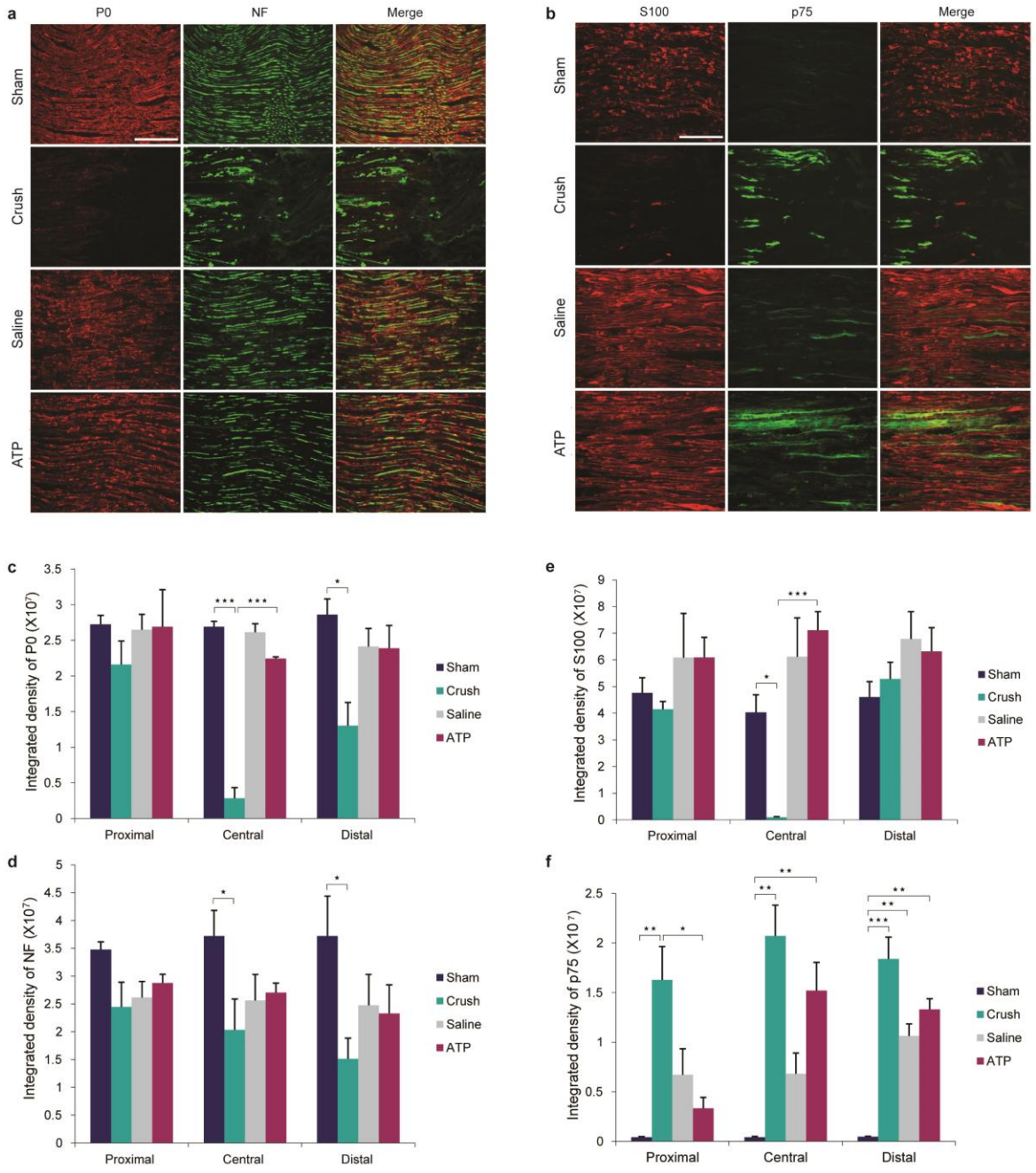


Figure 3-11 Morphological changes of sciatic nerves after nerve crush or single intraneural injections of saline or ATP. The sciatic nerve was removed three days after treatments (sham operation, sciatic nerve crush, injections of saline or 150 μM ATP into sciatic nerve). **a** and **b**, Immunoreactivity of myelin protein P0 (red in **a**), axon marker neurofilament 200 (NF, green in **a**), Schwann cell marker S100 (red in **b**), and $p75^{NTR}$

(green in **b**) in the central treatment region in sciatic nerve. Scale bars, 100 μm. **c** – **f**, Quantification of the four markers in the proximal, central, and distal regions of the treatments. Data are presented as means ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, one-way ANOVA with post-hoc comparisons and Bonferroni correction, n = 3.

3.5 Discussion

Since a conditioning lesion, which switches neurons to an active 'regeneration mode', is not clinically applicable, we have tested whether ATP injection into a sciatic nerve can mimic the growth-promoting effects of a conditioning lesion. In this chapter, we first demonstrated that intraneural injection of ATP could mimic the effects of a conditioning lesion in both in vitro and in vivo models. Enhanced neurites outgrowth (in vitro) after ATP injection was comparable with the results of the sciatic nerve crush group. However, our in vivo data have shown that ATP injection was not as potent as sciatic nerve crush in inducing axonal regeneration after dorsal column injury (transection).

It is suggested that signals transported back from the (peripheral) axonal injury site modulate the cell body response to injury, which leads to the activation of the intrinsic neuronal ability to regenerate and repair (Hanz and Fainzilber, 2006); several neuropoietic cytokines, transcription factors, kinases and growth associated protein are implicated in this retrograde signalling.

Briefly, a conditioning lesion causes upregulation of the cytokines LIF, IL-6 and CNTF (Cao et al., 2006, Rajan et al., 1995, Sendtner et al., 1997). These neuropoietic cytokines act through its receptor complex with a shared protein gp130, and its downstream signalling mediators are the JAK-STAT3 cascade (Taga and Kishimoto, 1997, Miao et al.,

2006). Upon a conditioning lesion, phosphorylated (activated) STAT3 is translocated to the nuclei in DRG neurons and initiate gene expression for axonal regeneration: STAT3 responsive genes include SOCS3 and pro-regenerative genes (Schweizer et al., 2002, Qiu et al., 2005, Neumann and Woolf, 1999, Dziennis and Alkayed, 2008).

On the other hand, other transcription factors such as ATF3 (a basic leucine zipper transcription factor) and cJun (immediate-early AP-1 transcription factor) are also activated in DRG neurons following a conditioning lesion (Tsujino et al., 2000, Broude et al., 1997, Murray-Rust et al., 2001). Several kinases, such as MAP kinases Erk1 and Erk 2 (Obata et al., 2004), as well as Jnk, are activated in cell bodies in response to the peripheral axotomy (Kenney and Kocsis, 1998). Downstream events affected by axotomy-activated kinases include upregulation or activation of transcription factors. For example, lesion-induced Jnk induces the upregulation and phosphorylation of the transcription factors c-Jun, JunD and Fos, and also the translocation of ATF3 into the nuclei, leading to changes of gene expression in the injured neuron, with upregulation of regeneration-associated genes (Kenney and Kocsis, 1998, Lindwall et al., 2004, Lindwall and Kanje, 2005, Seijffers et al., 2006). Interestingly, ATF3 enhances the c-Jun mediated neurite growth, while c-Jun is able to induce ATF3 expression, indicating the interrelationship between them (Pearson et al., 2003).

In addition, it is known that axonal regeneration involves expressions of regeneration-associated genes (RAGs) such as GAP43, Cap23, Arg1, and Sprr1a (Sun and He, 2010, Bisby, 1988). GAP43 is generally considered a growth indicator, since high levels of GAP43 are associated with increased neuronal growth capacity. GAP43 is expressed in a low percentage of DRG neurons in normal animals, but after sciatic nerve injury GAP43 expression in ipsilateral DRG neurons is significantly increased. The

expression of GAP43 is increased in DRG neurons upon conditioning lesion (Mulloy and Linhardt, 2001, Hu-Tsai et al., 1994) and is considered to be a marker for axonal regeneration (Schreyer and Skene, 1993, Skene, 1989). Interestingly, STAT3 is associated with the GAP43 expression during neurite outgrowth (Qiu et al., 2005), indicating their positive relationship in axonal regeneration.

Our data showed that the ATP-mediated conditioning-like effects are supported by upregulation of neuropoietic cytokines, transcription factor STAT3 and GAP43, all of which are closely related to the effects of the conditioning lesion. Our results suggest that CNTF and IL-6 were upregulated in the sciatic nerve and DRG after ATP injections and the level of these neuropoietic cytokines in the ATP injection group was comparable to the sciatic nerve crush group. Also, our data showed activation of STAT3 following ATP injection, suggesting that upregulation of neuropoietic cytokines and the consequent activation of JAK/STAT3 pathway play an important role in ATP-mediated conditioning-like effects. In addition, our results showed that GAP43 was upregulated after ATP injections, further supporting the notion that ATP promotes the growth capacity of the DRG neurons. However, pSTAT3 and the GAP43 level of the ATP group were significantly lower than those of the sciatic nerve crush group.

Moreover, other transcription factors such as ATF3 and pcJun, being known to be involved in the conditioning lesion phenomenon, did not increase in response to ATP injections. Only a moderate increase of ATF3 and pcJun was observed which was caused by the injection itself than the molecular action of ATP, since there was no difference between the saline and ATP injection groups. The data suggests that these transcription factors are not involved in ATP-mediated conditioning-like effects.

Analysis of the activation of STAT3 in subpopulations of DRG neurons showed that significantly higher percentage of $N52^+$ neurons expressed pSTAT3 than $IB4^+$ neurons, and similar results were obtained in the analysis of the expression of GAP43, suggesting that ATP injection induced STAT3 activation and GAP43 expression in both myelinated $N52^+$ and unmyelinated $IB4^+$ subpopulations of DRG neurons.

Morphological studies of the sciatic nerve using immunohistochemical analysis suggested that ATP injection did not significantly affect the immunoreactivity of the P0 (peripheral myelin), S100 (Schwann cells) or NF (neurofilaments), while sciatic nerve crush significantly reduced all of them. Sciatic nerve crush also significantly increased the expression of the $p75^{NTR}$, a marker for de-differentiated Schwann cells during Wallerian degeneration. ATP injection increased the expression of the $p75^{NTR}$ in central and distal regions of the sciatic nerve, although to lesser degrees than sciatic nerve crush, indicating that the injection procedure itself caused little Wallerian degeneration at the injection site. Since we have observed morphological changes after ATP injection, we next determined the sensory and motor function of the sciatic nerve after ATP injection via behaviour studies. This will be discussed in the next chapter.

Taken together, the results of our experiments demonstrate that intraneural injection of ATP can promote central sensory axonal regeneration mimicking a conditioning lesion. However, it is still unclear whether ATP is an exact initiating molecule of the conditioning lesion as the degrees and pattern of regeneration appear differently. It is possible that a higher concentration of ATP may be present in the extracellular space after sciatic nerve crush than our injected concentration. Using different (higher) concentrations of ATP might induce more effective regenerative responses. Nevertheless, it is still useful to use this ATP

injection method to induce enhanced axonal regeneration after CNS injury since the conditioning lesion itself cannot be used clinically.

Since the effect of ATP injection was not as potent as sciatic nerve crush, a logical next step was to find out ways of increasing its potency. Using more potent, selective and degradation resistant agonists could be one way to achieve this. Another way might be using ecto-ATPase inhibitors such as PV4 with ATP to increase the extracellular concentration of ATP (Melani et al., 2012). Furthermore, combined therapy with other strategies, such as making CNS environments more permissive using PSA or myelin inhibitor antibodies as we previously discussed in Chapter 1, could also be used to exert synergic effects. However, we firstly wanted to explore the further possibilities of using ATP injection as a single agent therapy, so we decided to repeat the ATP injection one more time to sustain the intrinsic growth capacity of the sensory neurons, without any alteration of the CNS environment. The results will be discussed in the next Chapter.

CHAPTER 4 - ENFORCING THE STIMULATORY EFFECT OF SINGLE ATP INJECTION BY A SECOND ATP INJECTION

4.1 Abstract

Since a conditioning lesion is not a clinically feasible approach for promoting axonal regeneration, we explored an alternative treatment and found that injection of ATP into a peripheral nerve significantly promoted the growth of sensory axons in the injured spinal cord as we discussed in the last chapter. Although the ATP injection mimicked conditioning effects, it was to a lesser degree than the effects of a conditioning lesion. Since the application of a second conditioning lesion (repriming) was able to sustain intrinsic growth state of sensory neurons (Neumann et al., 2005), we applied a second ATP injection to examine whether it would be more effective than a single injection. Strikingly, a second ATP injection (ATP/ATP) triggered a 166-fold increase in the number of sensory axons growing into the lesion centre compared with the double saline injection group (saline/saline). Some of the regenerating axons of the ATP/ATP group extended into the rostral spinal cord, while no axon in the ATP/saline and saline/saline groups grew into the rostral spinal cord. Double ATP injection is therefore found to be much more potent than a single conditioning lesion in enhancing sensory axonal regeneration. Also, after intraneural ATP injection, motor and sensory functions of the injected sciatic nerve returned to the baseline level within 6 days, suggesting no long-term adverse effect. Our findings indicate that intraneural ATP injection could be a potential therapeutic approach to treat patients with spinal cord injury.

4.2 Introduction and aims

Although we found that a single intraneural ATP injection into the sciatic nerve can promote central sensory axon regeneration, the number of regenerating axons growing into

the lesion cavities was still relatively low (lower than a conditioning nerve crush), and no axon grew out of the lesion cavities and into the rostral spinal cord. One reason for the low efficacy of a single ATP injection may be that activated transcription factors such as STAT3 in DRG neurons decrease in a few days (Qiu et al., 2005).

It was reported previously that application of a second conditioning lesion (repriming) sustained the intrinsic growth capacity of sensory neurons and significantly enhanced sensory axonal regeneration, much more effectively than a single one (Neumann et al., 2005). Neumann and her colleagues achieved axonal regrowth significantly rostral to the lesion without altering the CNS environment (e.g., addition of growth factors) and concluded that to achieve long distance regeneration, it is crucial not only to induce neurons into a 'growth' mode but also to sustain this state. Therefore, we postulated that a second ATP injection a week after the initial one would also be able to sustain the regeneration capacity of injured sensory axons, possibly making injured axons regenerate over a longer distance.

Also, as we observed little morphological changes (Wallerian degeneration) at the injection site in the previous chapter, we designed behavioural tests to examine sensory and motor functions of the sciatic nerve after intraneural injection of ATP.

The aims of this chapter are as follows:

a) To examine whether a second ATP injection into the sciatic nerve one week after the first one is more effective than a single ATP injection.
b) If so, to investigate whether the activation of transcription factor STAT3 and the expression of GAP43 are sustained for a longer period of time after a second ATP injection.

c) To examine whether ATP injections affect the motor and sensory functions of the sciatic nerve.

4.3 Methods

Three groups of animals (8 per group) were subjected to dorsal column transection and then two groups were administered intraneural ATP injections and one group was administered saline injections. One week later, one ATP injection group was administered a second intraneural ATP injection (ATP/ATP group). The other ATP injection group received a saline injection as a single ATP injection control (ATP/saline group), while the saline injected animals were administered a second saline injection as control (saline/saline group). All the other experimental procedures were the same as those of single treatment experiments. Behavioural studies including mechanical sensitivity test, thermal sensitivity test, footprint and grid walking were performed for three weeks after the initial injections. After the behavioural studies, the animals were sacrificed and DRG were taken to check the activation of transcription factor STAT3 and expression of GAP43. Six weeks after the dorsal column transection and the initial sciatic nerve injection, the left sciatic nerve was exposed at hip level and 5 µl of 1% CTB were injected to trace the axonal regeneration in the dorsal column. Further details can be found in Chapter 2. Figure 4-1 summarizes the timeline of the experimental procedures in Chapter 4.

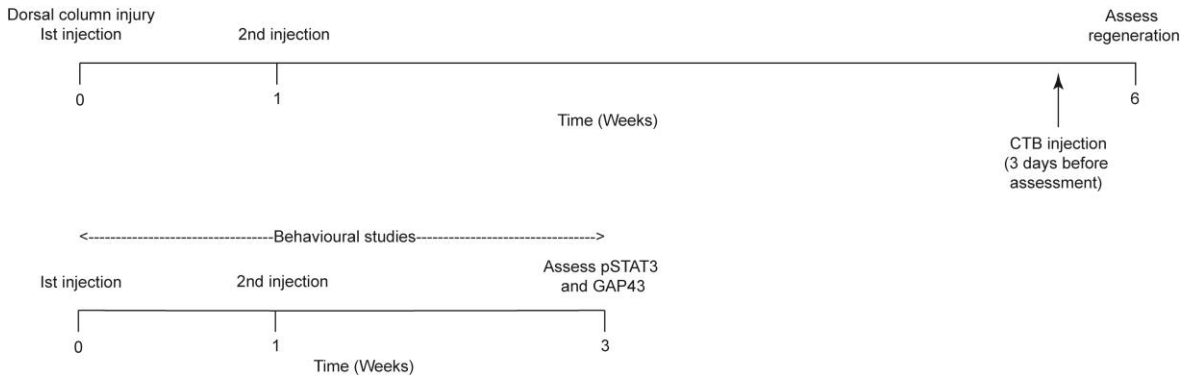


Figure 4-1 Timeline of the experimental procedures.

4.4 Results

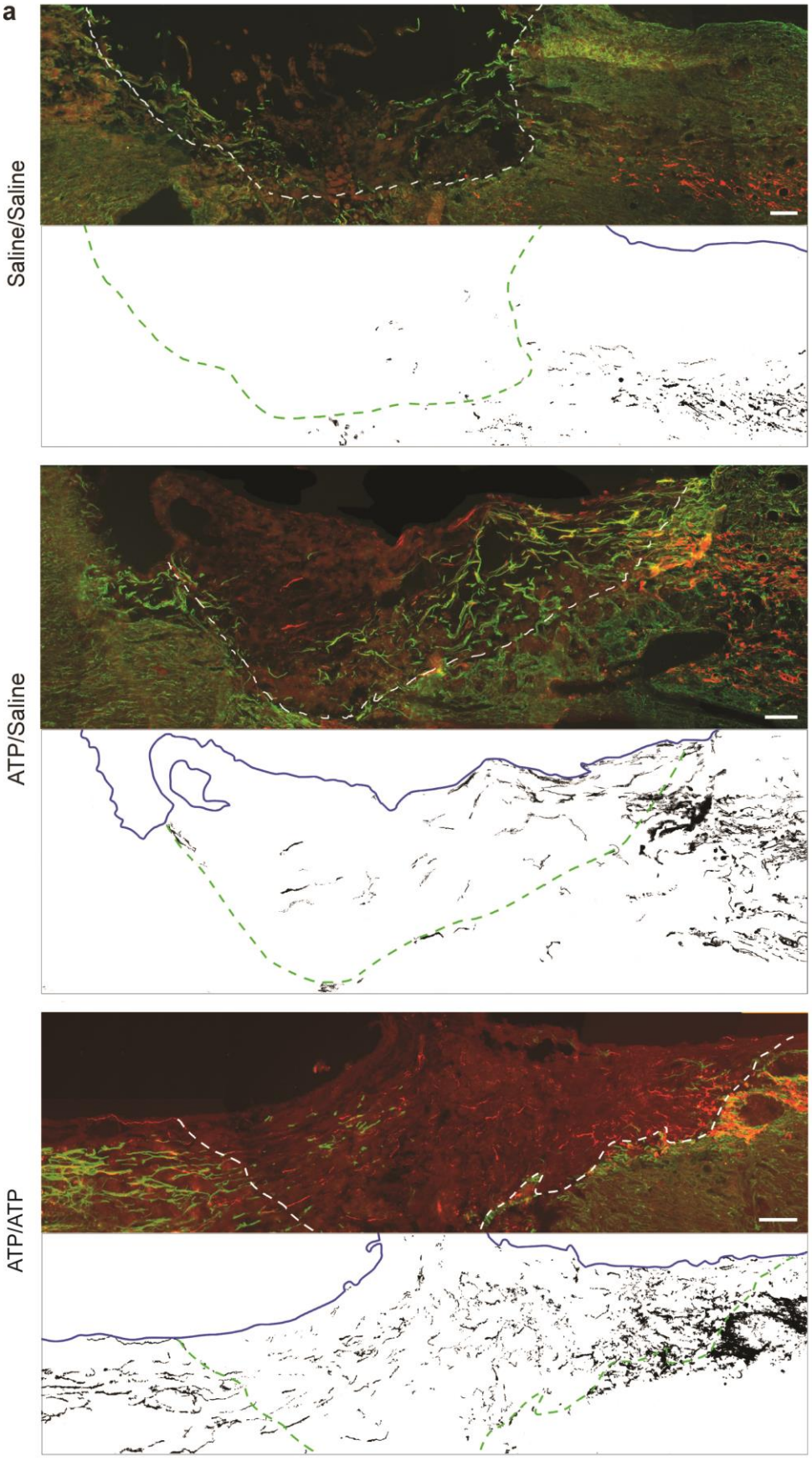
4.4.1 Double ATP injections enhance axonal regeneration beyond the lesion cavity

Double ATP injection is found to be much more potent than a single ATP injection in enhancing sensory axonal regeneration. For a more accurate comparison of the CTB-labelled axons inside the lesion among the three groups, the lesion site was divided into three regions: caudal, centre, and rostral (Figure 4-2). In the saline/saline group, only limited numbers of CTB-labelled axons were observed at the caudal region of the lesion (22 ± 4), very few axons were observed at the lesion centre, and no axons were observed at the rostral region. In the ATP/saline group, most of the CTB-labelled axons were observed at the caudal region of the lesion (146 ± 25), far fewer at the lesion centre (43 ± 10) and even fewer at the rostral region (7 ± 3). However, most strikingly, in the ATP/ATP group, 498 ± 88 CTB-labelled axons were observed at the caudal region of the lesion, 415 ± 88 CTB-labelled axons at the lesion centre, and 198 ± 60 CTB-labelled axons at the rostral region of the lesion. The number of CTB-labelled axons present in the lesion centre in the ATP/ATP

group was 10 times greater than that in the ATP/saline group, and 166 times greater than that in the saline/saline group.

Some of the regenerating axons extended into the degenerating dorsal column of the rostral region of the spinal cord as far as 1.75 mm in the ATP/ATP group, while no axon in the other groups (ATP/saline, saline/saline and even sciatic nerve crush) grew into the rostral spinal cord. The results indicate that a second ATP injection (ATP/ATP) triggered remarkable increase in the regrowth of sensory axons compared either with the saline/saline group or ATP/saline group.

No CTB staining was visible in the transverse sections of upper cervical spinal cord indicating the dorsal column transection in thoracic level was complete (Figure 4-3, representative of ATP/Saline group).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Saline/Saline
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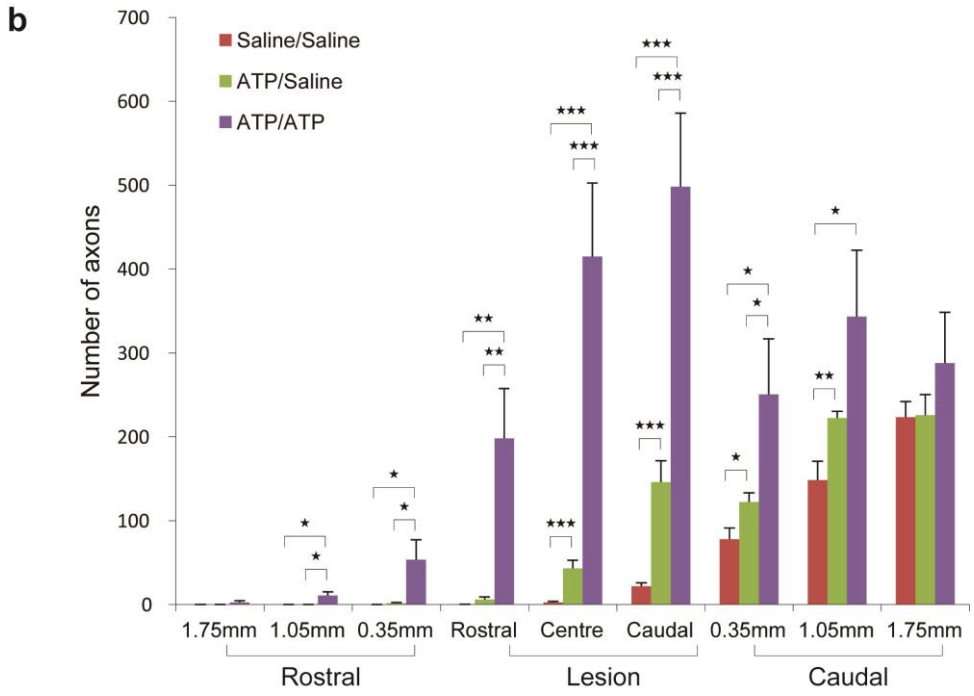

Figure 4-2 Double intraneural injection of ATP promotes profound axonal regeneration after dorsal column transection. a, Photomicrographs showing CTB-labelled sensory axons (red) growing around and into the lesion cavity (defined by green GFAP immunostaining, and marked by dashed lines) in the spinal cord sagittal sections from saline/saline, ATP/saline, and ATP/ATP double injection groups. Camera lucida drawings corresponding to the same photomicrographs were used to illustrate the CTB-labelled axons inside and around the lesion cavity. Caudal is to the right, and rostral is to the left. Scale bar = 100 µm. **b**, Quantification of the numbers of CTB-labelled axons in the three regions (caudal, centre, and rostral) of the lesion cavity, and at the rostral and caudal sides of the lesion cavity. Data are presented as means ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with Bonferroni's post hoc test, n=8.

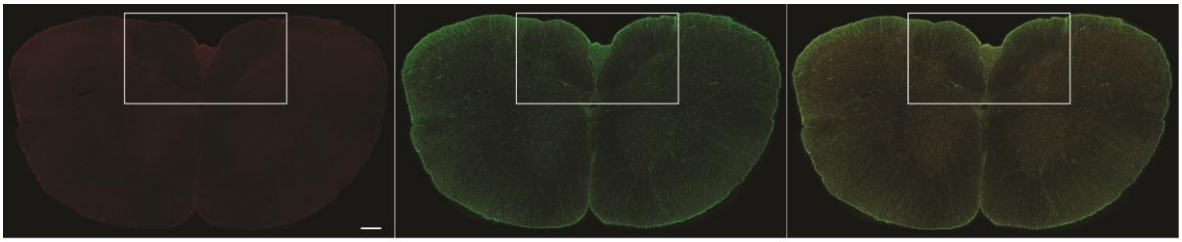

Figure 4-3 CTB staining in upper cervical spinal cord. Photomicrographs showing no CTB-labelled sensory axons (red) in the upper cervical spinal cord transverse sections. Boxed area: dorsal column part of spinal cord including fasciculus gracilis and fasciculus cuneatus. Scale bar, 200 μm.

4.4.2 Double ATP injections sustained activation of STAT3

To investigate whether a second ATP injection can sustain the activation of STAT3 in DRG, the rats were sacrificed three weeks after the initial injection and DRG were removed for staining of pSTAT3. Double saline injections (Saline/Saline) did not induce a significant increase in the percentage of $pSTAT3^+$ neurons (18 ± 2 %), compared with double sham-operated animals (Sham/Sham, 12 ± 1%; Figure 4-4). In the ATP/saline group, $pSTAT3^+$ neurons were significantly increased to 30 ± 1%, compared with the saline/saline group (P = 0.04, one-way ANOVA test). However, double ATP injections (ATP/ATP) induced a significantly higher increase in the percentage of $pSTAT3^+$ neurons (45 ± 5%), compared either with ATP/saline group (P = 0.006, one-way ANOVA test) or Saline/Saline group (P = 2×10^{-4}, one-way ANOVA test). This indicates that the sustained activation of STAT3 may contribute to the significantly enhanced axon growth in the injured spinal cord after double ATP injection.

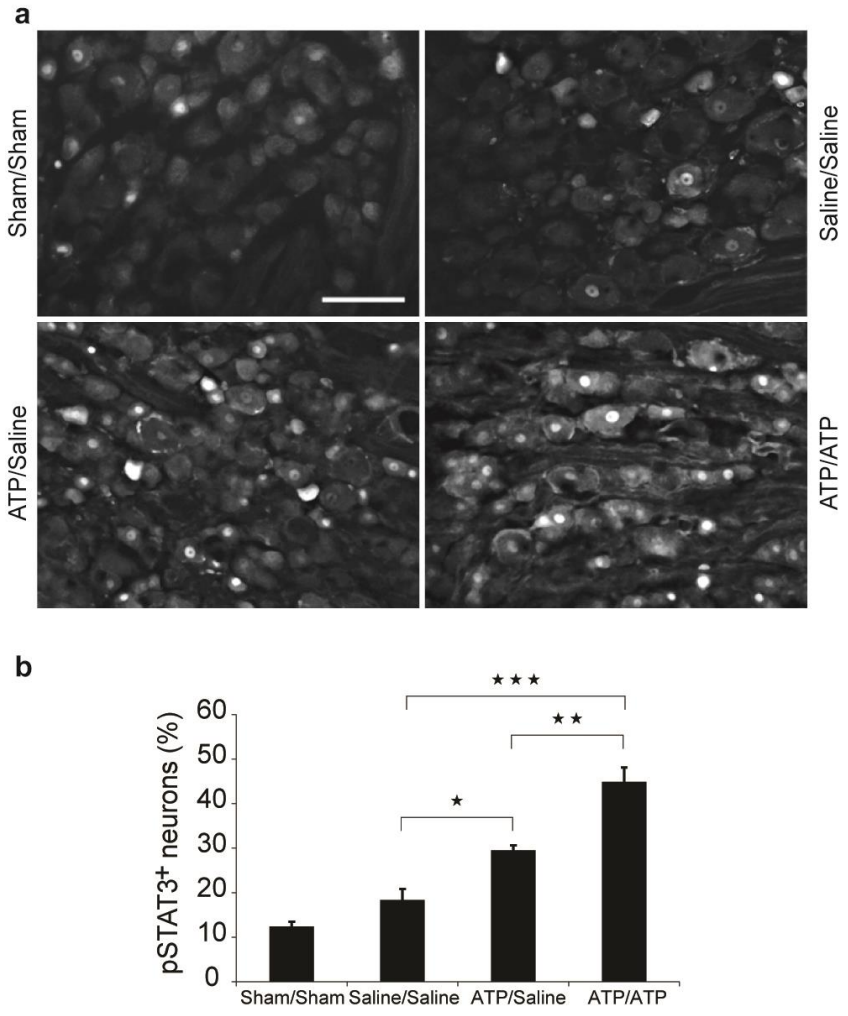

Figure 4-4 Double intraneural ATP injection induces sustained activation of STAT3 in DRG neurons. a, Microphotographs of immunoreactivity for phosphorylated STAT3 (pSTAT3) in L4–5 DRG neurons from the four groups of rats subjected to double sham operation, saline/saline double injection, ATP/saline double injection, and ATP/ATP double injection into the sciatic nerve. Scale bar, 100 μm. **b**, Quantification of neurons with nuclear profiles containing $pSTAT3^{+}$ neurons. The number of neurons with nuclei containing pSTAT3 immunoreactivity is expressed as percentage of the total number of

neurons with visible nuclei (stained by DAPI). Data are means ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with Bonferroni's post hoc test, n = 8.

4.4.3 Double ATP injections sustain expression of GAP43

To investigate whether a second ATP injection can sustain the expression of GAP43 in DRG, the rats were sacrificed three weeks after the initial injection and DRG were removed for staining of GAP43. Double saline injections (Saline/Saline) did not induce a significant increase in the percentage of $GAP43^+$ neurons (27 ± 1 %), compared with double sham-operated animals (Sham/Sham, 23 ± 2%; Figure 4-5). In the ATP/saline group, $GAP43^+$ neurons were significantly increased to 42 ± 1%, compared with the saline/saline group ($P = 0.008$, one-way ANOVA test). However, double ATP injections (ATP/ATP) induced a significantly higher increase in the percentage of $GAP43^+$ neurons (60 ± 4%), compared either with the ATP/saline group ($P = 0.003$, one-way ANOVA test) or Saline/Saline group ($P = 4\times10^{-5}$, one-way ANOVA test). These data suggest that the sustained expression of GAP43 may contribute to the significantly enhanced axon growth in the injured spinal cord after double ATP injection.

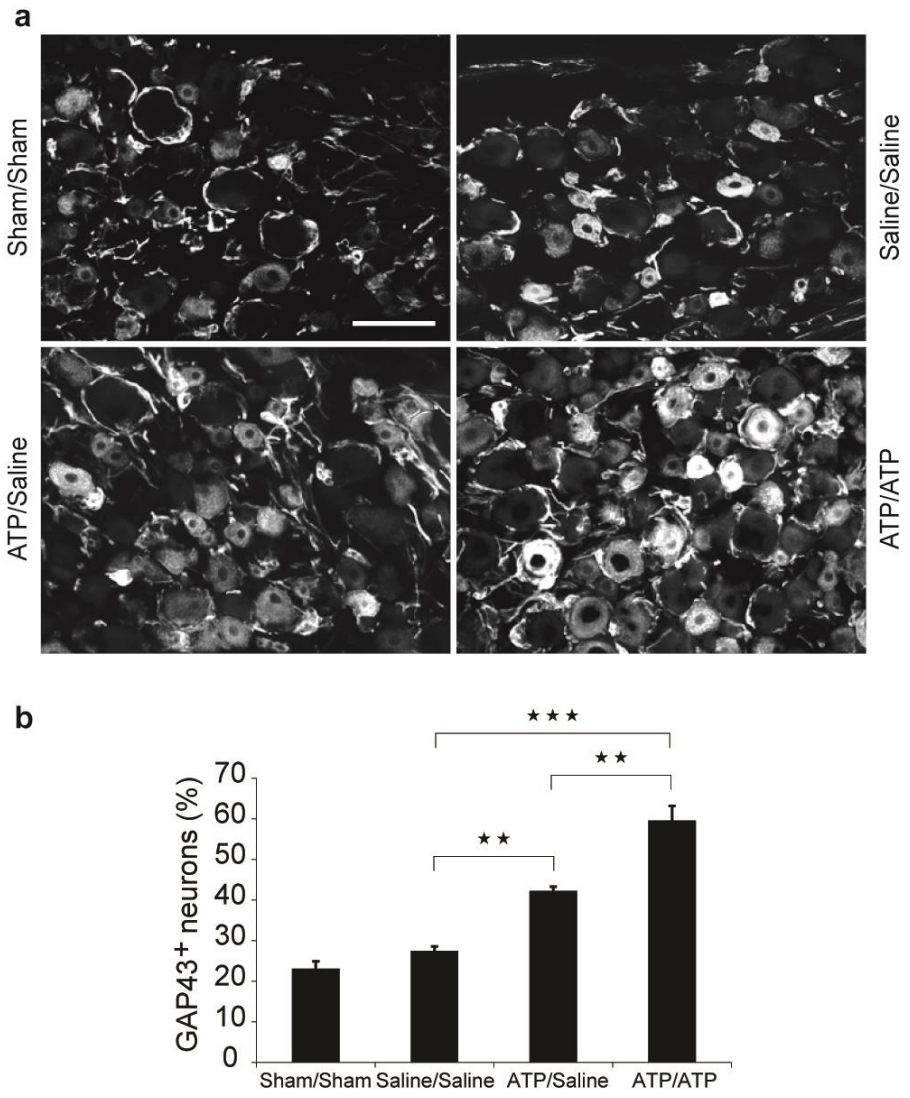

Figure 4-5 Double intraneural ATP injection induces sustained expression of GAP43 in DRG neurons. a, Microphotographs of immunoreactivity for GAP43 in L4–5 DRG neurons from the four groups of rats subjected to double sham operation, saline/saline double injection, ATP/saline double injection, and ATP/ATP double injection into sciatic nerve. Scale bar, 100 μm. **b**, Quantification of $GAP43^+$ neurons. The number of neurons with GAP43 immunoreactivity in the cytoplasm is expressed as a percentage of the total number of neurons with visible nuclei (stained by DAPI). Data are means ± SEM. ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with Bonferroni's post hoc test, n = 8.

4.4.4 The effect of intraneural injection of ATP on motor and sensory functions of the sciatic nerve

To assess whether intraneural ATP injection can be clinically applied to patients without having adverse effects on sciatic nerve functions, we carried out behavioural studies to assess the motor and sensory functions of the hindlimbs following sciatic nerve injections. Four groups of rats (Sham/Sham, Saline/Saline, ATP/Saline, ATP/ATP; five rats per group) were subjected to tests for hindpaw withdrawal upon mechanical or thermal stimulation, footprint analysis and grid walking after the treatments. Double sham operation (Sham/Sham) group and double saline injection (Saline/Saline) groups were used as controls.

First of all, single and double injections of either saline or ATP did not cause any significant change in the mechanical sensitivity of hindpaws, measured by hindpaw withdrawal threshold, during the 20 day testing period, compared with either the baseline values or the sham operated control on each test day (Figure 4-6). Second, mild thermal hyperalgesia, measured by hindpaw withdrawal latency, was observed 2 days after initial ATP injection (Figure 4-7), compared with either the baseline values (P = 0.002, two-way ANOVA test) or the sham operated control on day 2 (P = 0.01, two-way ANOVA test). However, the thermal withdrawal latency of hindpaws returned to baseline level on day 6. Second intraneural injection of ATP or saline had no effect on thermal thresholds of the hindpaw.

Similarly, on day 2 after an initial injection of either saline or ATP, footprint analysis showed that the distance between the 1st and 5th toe, an index for sciatic nerve motor function, was significantly reduced (Figure 4-8), compared with either the baseline

values (P = 0.004 in Saline and P = 0.002 in ATP group, two-way ANOVA test) or sham operated control on day 2 (P = 0.002 in Saline and P = 0.005 in ATP group, two-way ANOVA test). However, it returned to baseline level on day 6 and remained at that level throughout the testing period (until day 20). The second intraneural injection of ATP or saline had no effect on the distance of the 1st to 5th toe. For the grid walking test, the initial ATP injection induced a significant increase in hindlimb foot slips on day 2 post-injection, compared with the baseline values (P = 0.043, two-way ANOVA test). However, the number of foot slips returned to baseline level on day 6. Second injection of ATP or saline did not cause a significant increase in the number of foot slips in grid walking (Figure 4-9). The results showed that injection of ATP or saline induced an acute temporary mild injury to the sciatic nerve.

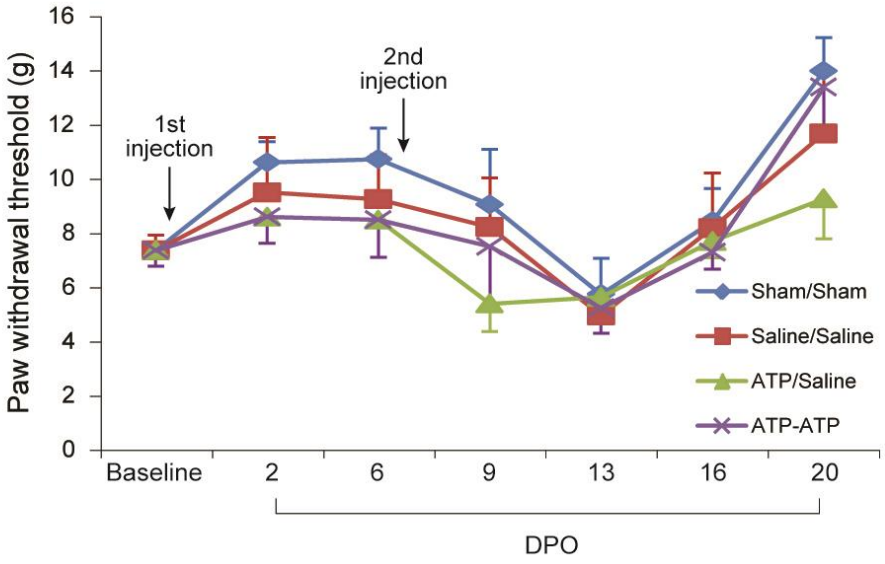

Figure 4-6 The effects of double intraneural injection of ATP on mechanical sensitivity. Mechanical sensitivity was assessed by measuring paw withdrawal threshold using Von Frey hairs. Animals were tested three times to obtain baseline values before the

first intraneural injection of ATP or saline, followed by a second injection of ATP or saline 7 days later. For the sham-operated control, the sciatic nerve of the rats was exposed without any treatment. Data are means ± SEM. Two-way ANOVA with Bonferroni's post hoc test, n = 5.

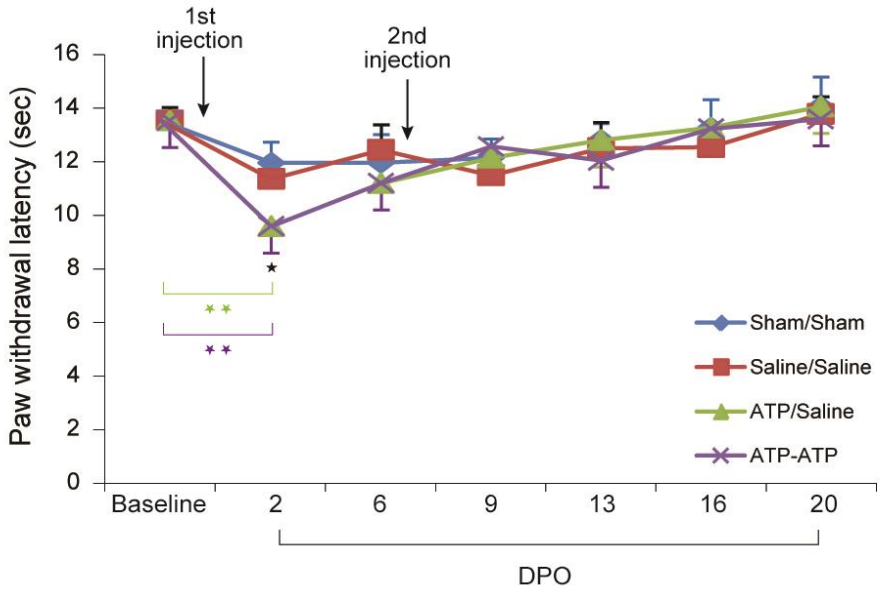

Figure 4-7 The effects of double intraneural injection of ATP on thermal sensitivity. Thermal hypersensitivity was assessed using the Hargreaves' method of the heat plantar test. Animals were tested three times to obtain baseline values before the first intraneural injection of ATP or saline, followed by a second injection of ATP or saline 7 days later. For the sham-operated control, the sciatic nerve of the rats was exposed without any treatment. Data are means ± SEM. * $P < 0.05$; ** $P < 0.01$. Coloured stars are compared with the baseline for the same group and black stars are compared with Sham/Sham group on the same test day. Two-way ANOVA with Bonferroni's post hoc test, n = 5.

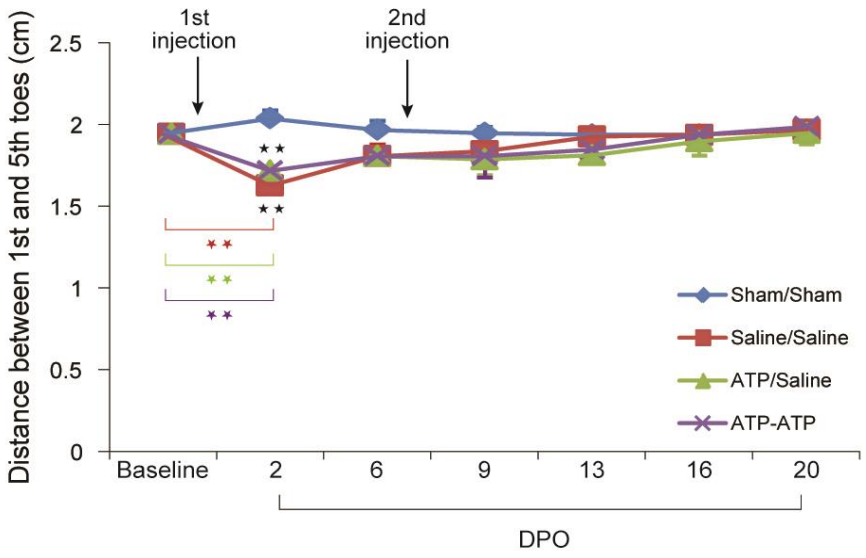

Figure 4-8 The effects of double intraneural injection of ATP on 1st to 5th toe spread. The distance between the 1st and 5th toe on the footprint was used as an indicator of the sciatic nerve function. Animals were tested three times to obtain baseline values before the first intraneural injection of ATP or saline, followed by a second injection of ATP or saline 7 days later. For the sham-operated control, the sciatic nerve of the rats was exposed without any treatment. Data are means ± SEM. ** $P < 0.01$. Coloured stars are compared with the baseline for the same group and black stars are compared with Sham/Sham group on the same test day. Two-way ANOVA with Bonferroni's post hoc test, n = 5.

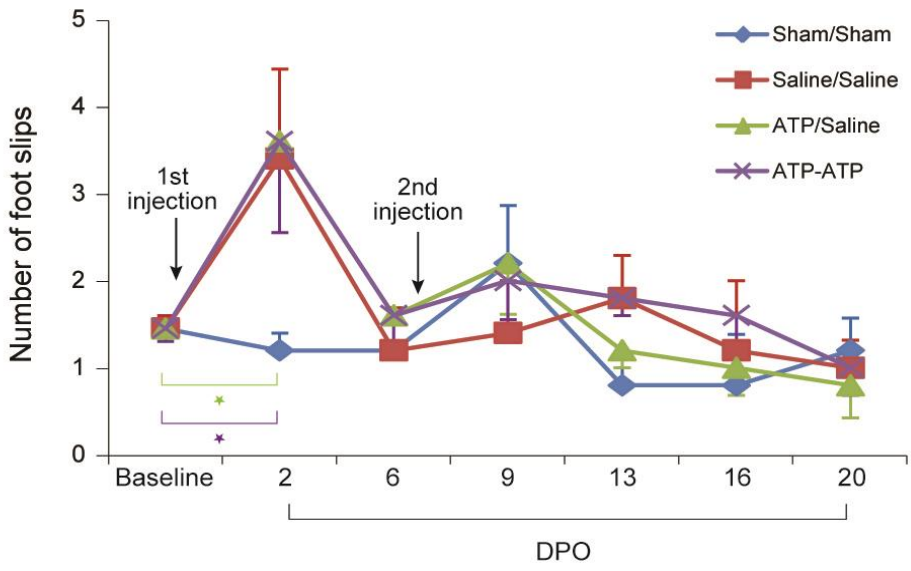

Figure 4-9 The effects of double intraneural injection of ATP on hindlimb foot slips in grid walking. Foot slips in grid walking were analyzed to evaluate sensorymotor coordination after intraneural injection of ATP. Animals were tested to obtain baseline values before the first intraneural injection of ATP or saline, followed by a second injection of ATP or saline 7 days later. For the sham-operated control, the sciatic nerve of the rats was exposed without any treatment. Data are means ± SEM. * $P < 0.05$, compared with the baseline for the same group, two-way ANOVA with Bonferroni's post hoc test, n = 5.

4.5 Discussion

One of the main reasons why adult CNS neurons in mammals fail to reconnect their targets after injury is that the nervous system is significantly larger in the adult than during development, so adult neurons have to regenerate over a much longer distance (Liu et al., 2011). In fact, in order to re-establish the functional circuits of the CNS after injury, axons need to regenerate over a long enough distance to reach their functional targets, far beyond

the lesion cavity. To achieve long distance anatomical regeneration, it is important to not only induce neurons to enter an enhanced 'regeneration' mode, but also sustain this growth state for a sufficient period of time (Neumann et al., 2005). Therefore, an obvious limiting factor for axonal regeneration in adults is the neuronal competence in sustaining intrinsic growth capacity for long distance regrowth.

As discussed in Chapter 3, ATP injection induced axonal regeneration after dorsal column transection mimicking the conditioning lesion (sciatic nerve crush). A limitation was that the regenerating axons only grew into the lesion cavity, but no axons grew out to the rostral regions of spinal cord and therefore, it is far from achieving the goal of functional recovery.

Repeated administration of a conditioning lesion was found to be effective in sustaining the intrinsic growth state of sensory neurons and allowed axons to regenerate over a much longer distance (Neumann et al., 2005). Therefore, we postulated that double ATP injections should be more effective than a single injection in making axons regenerate over longer distances towards their rostral targets. The data derived from our experiments in Chapter 4 demonstrates that double intraneural injection of ATP can promote central sensory axonal regeneration beyond the lesion cavity and into the rostral regions of the spinal cord as far as 1.75 mm. It was significantly more effective than not only a single ATP injection, but also a single conditioning lesion (either sciatic nerve crush in our experiments in Chapter 3 or transection by others (Neumann and Woolf, 1999)), and indeed many other treatments that failed to promote growth into the rostral spinal cord.

Others also reported the axonal regrowth beyond the lesion cavity into the rostral spinal cord. Lu et al. (2004) used the combined therapy of intragaglionic injection of cAMP,

bone marrow cell transplantation at the lesion site, and NT-3 injections near the tips of the injured axons and 1.5mm rostral to the lesion site. Their data demonstrated that axons grew beyond the lesion site at a range of around 0.5 - 0.7mm. Furthermore, repeated conditioning lesions (sciatic nerve transection, 'repriming') from Neumann and colleagues (2005) resulted in axonal regrowth beyond the lesion of around 0.7 mm after 6-8 weeks post repriming. However, the former is a combined therapy and the latter is clinically impractical. The value of our data is that the method used is a single agent therapy and also more clinically applicable. In this aspect, our results, that axons grew as far as 1.75 mm rostral to the lesion site of the spinal cord, are not comparable with any other treatments to date.

Our study is the first to demonstrate that injection of a bioactive compound into a peripheral nerve, without any alteration of the CNS environment or any other combined therapeutic approaches, can achieve such a profound effect in promoting axonal regeneration. We anticipate that if ATP injection is combined with other approaches, such as providing cellular bridging using transplanted cells (Leavitt and Freire, 2001, Spyracopoulos and Sykes, 2001), blocking the myelin-associated inhibitors and their signalling pathways (Unger, 2001, Lakshmi and Brudvig, 2001), degrading CSPG (Mattos and Ringe, 2001) or making CNS more permissible by over-expressing polysialic acid (Zhang et al., 2007b), then an even more profound axonal regeneration may be achieved.

Although we have demonstrated that double intraneural ATP injections can promote axons growing into the rostral spinal cord, the axons only grew a finite distance which was still far from their functional targets and the axon numbers that reached the rostral spinal cord were still low. Presumably, this is largely due to the narrow time window of the regenerative process, supported by the enhanced intrinsic growth capacity after ATP

injections. Once axons pass the glial scar, they are no longer affected by the inhibitory molecules from scar tissue, even though myelin associated inhibitors and CSPG are still present in the degenerating dorsal column. It is highly possible that axons can grow further if the growth mode can be sustained for a longer period. As full development of glial scar takes several weeks (Fawcett and Asher, 1999), it would be worth repeating ATP injections a few more times before that to further maintain the time window of regeneration for even longer distance regeneration and to allow more axons to participate in the regenerative process.

To carefully assess the possibilities of clinical use of ATP injections, behavioural studies to test motor and sensory functions of the sciatic nerve were performed (Chapter 4) and morphological changes in the sciatic nerve were observed (Chapter 3). The results were that the effects on both sensory and motor functions of the sciatic nerve were temporary as they recovered to the basal level within a few days (Day 2-6) and the morphological changes of the sciatic nerve were very mild. There were no observable signs of pain or suffering in the animals during the testing period. These data indicate that intraneural injection of ATP remains a potential therapeutic method for clinical use without significant long-term adverse effects.

An alternative way of delivering ATP into the sciatic nerve may be considered to minimize the adverse effects of the intraneural injection method. A chronically implanted drug delivery system consists of a nerve cuff and a subdermal injection dome, and has shown to be effective at delivering local anesthetics such as lidocaine directly to peripheral nerves, including the sciatic nerve, for many months (Pohlmeyer et al., 2009). Such a system could be used to deliver ATP (or other P2 agonists) into the sciatic nerve. However, unlike lidocaine, ATP may not be able to penetrate the perineurium by diffusion as ATP

has several negatively charged groups in a neutral solution. Chemical modification of ATP (or other P2 agonists) to render them able to cross the perineurium will also be needed to employ this delivery system.

In this study, spinal cord injury and ATP injection were performed at the same time, which will of course not occur in clinical practice. Therefore, the efficacy of ATP injection in promoting sensory axonal regeneration needs to be tested in subacute and chronic injury animal models. Furthermore, it will also be interesting to test whether ATP can activate the regeneration mechanism in other types of neurons such as motor neurons after injury.

What remains to do next is to determine the purinoceptor subtype(s) that mediate the profound regenerative effects of ATP. The identification of the purninoceptor subtype(s) that is responsible for the enhanced regeneration capacity of DRG neurons may lead to the development of more potent agonists than ATP. This will be discussed further in the next chapter.

CHAPTER 5 - EXPRESSION AND DISTRIBUTION OF PURINERGIC RECEPTORS IN SCIATIC NERVE, DRG AND SCHWANN CELLS

5.1 Abstract

In the previous two chapters, we have seen the effects of ATP injection on axonal regeneration after injury, especially in the case of double injection. We have also seen some pathways/molecules involved in the ATP-mediated enhanced growth capacity of neurons. However, we do not know which specific receptor(s) are responsible for this ATP-mediated axonal regeneration. As the first step towards the identification of the specific receptor(s), in this chapter all the receptor subtypes present on adult rat sciatic nerves (and DRG) were identified using qPCR and immunohistochemistry methods. Also, co-labelling with either neuronal marker PGP 9.5 or Schwann cells marker S100 further allowed the localization of receptors either on nerve fibres (neurons) or the Schwann cells. Using quantitative PCR and immunohistochemistry, we have found that most of the purinoceptor subtypes are expressed in sciatic nerve fibres and Schwann cells to various levels. In cultured Schwann cells, high levels of mRNA transcripts of P2X4, P2Y2 and P2Y13 were detected. Transcripts of P2Y2, P2Y13, P2Y14, A1, and A2a were dominant in sciatic nerve tissue. In DRG, transcripts of P2X3, P2Y2, A1 and A2a were predominant. At protein level, DRG neurons showed strong immunoreactivity of P2X1, P2X3, P2X4, P2X6 and A1 receptors, while satellite cells showed strong immunoreactivity of P2X7 and P2Y2 receptors. Axons in the sciatic nerve showed strong immunoreactivity of P2Y2 receptors, while Schwann cells showed strong immunoreactivity of P2X7, P2Y2, P2Y12 and P2Y13 receptors.

5.2 Introduction

Extracellular ATP can activate most of the seven P2X (ATP-gated cation channels) and eight P2Y (G-protein coupled) receptor subtypes. In addition, extracellular ATP is

degraded rapidly to ADP, AMP and adenosine, and these purines can also activate purinergic receptors such as P2Y1, P2Y12 and adenosine receptors.

P2X receptors expression in DRG has been well studied. High level of P2X3 receptor in a subset of rat DRG neurons was detected (Xiang et al., 1998, Chen et al., 1995, Vulchanova et al., 1998, Vulchanova et al., 1997) and also mRNAs and proteins for other P2X receptors were identified in DRG neurons at a lower level (Xiang et al., 1998, Kobayashi et al., 2005). Also, there is some evidence of the presence of P2Y or P1 receptors on rat DRG as well. While a high level of mRNA transcripts for P2Y1 and P2Y2 was identified in rat DRG neurons, transcripts for P2Y4 and P2Y6 were detected at a low level (Newton et al., 1999, Townsend-Nicholson et al., 1999). Others have reported the presence of the A2a receptor in large size DRG neurons in rats (Kaelin-Lang et al., 1998).

However, the expression of the purinergic receptors on peripheral nerves and Schwann cells in rats has been less well investigated to date. There are some reports that suggest the presence of the P2Y1 and P2Y2 receptors on rat Schwann cells (Liu et al., 2005, Mayer et al., 1998). Also, studies using analogues/antagonists suggested the presence of adenosine A2 receptors in rat sciatic nerve (Sheldon et al., 1996).

The aim of this chapter is to identify the purinergic receptors present in the sciatic nerve (and DRG) and to confirm whether they are present on nerve fibres/neuronal cell bodies or Schwann cells/satellite cells. This would be the first step in identifying the specific receptor subtype involved in ATP-mediated growth-promoting effects and to develop more potent drugs (agonists) to effectively promote axonal regeneration after injury.

5.3 Methods

Unperfused tissues (sciatic nerve and L4-5 DRG) from normal Wistar rats were used for extracting RNA for qPCR. qPCR was used to detect the level of transcripts of 18 purinergic receptor subtypes which have been cloned in rats to date. PFA perfused tissue (sciatic nerve and L4-5 DRG) from normal Wistar rats was used for immunostaining. The sciatic nerve and DRG were stained for each purinergic receptor subtype as well as with either neuronal marker PGP 9.5 or Schwann cells marker S100. The level of expression was determined by the naked eye following the criteria previously described (Xiang et al., 1998). The details can be found in Chapter 2.

5.4 Results

5.4.1 mRNA expression of purinergic receptors in sciatic nerve, DRG and Schwann cells

We used realtime PCR to quantify the transcripts for all known rat purinergic receptor subtypes (except P2Y11, which is not cloned in rats) in the sciatic nerve, DRG and Schwann cells. The results demonstrate the presence of transcripts of 18 purinergic receptor subtypes at different levels (Figure 5-1). Each primer pair gave one band with predicted size and the non-template (NT) control failed to show any band.

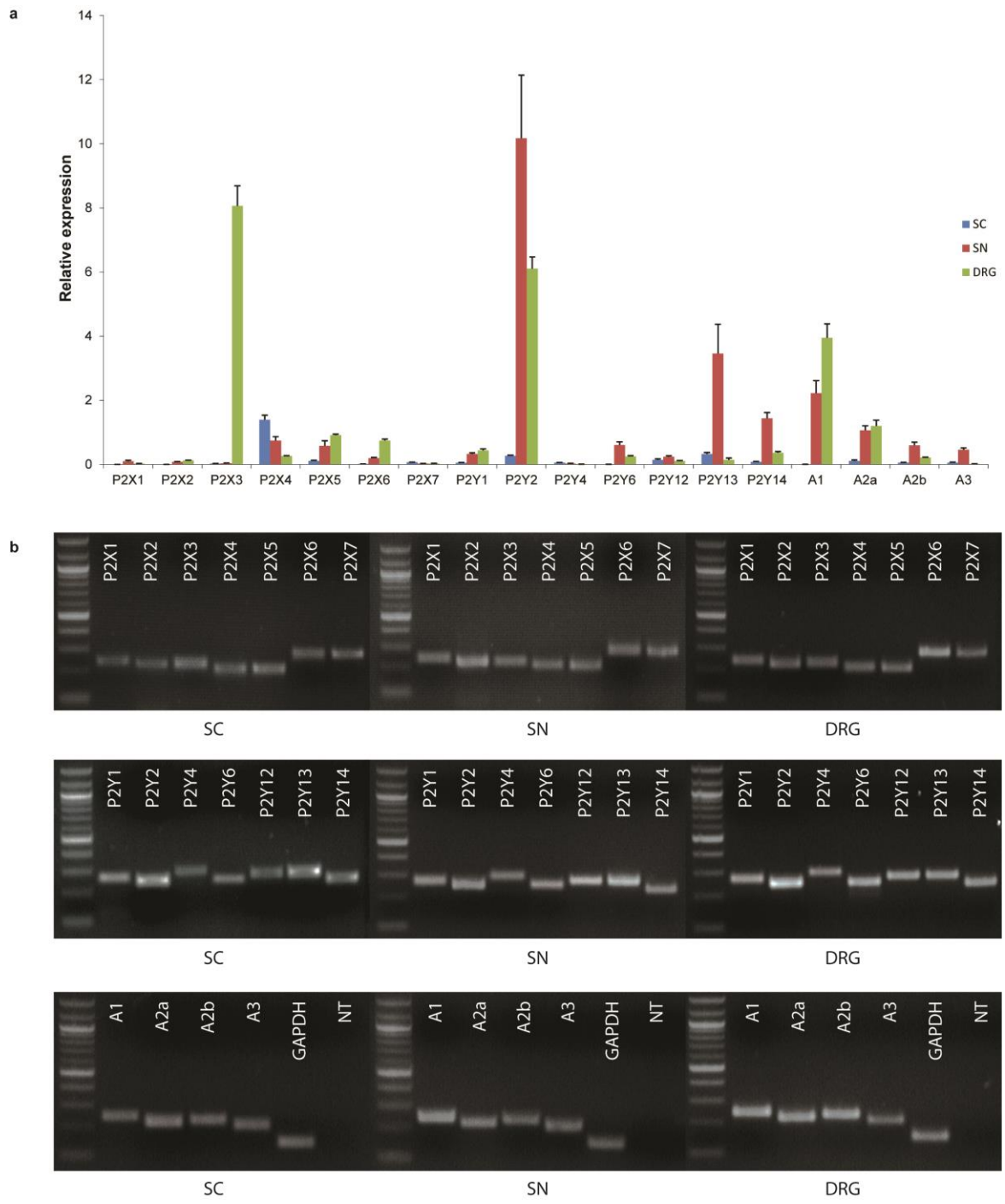

Figure 5-1 Relative level of expression of purinergic receptor mRNAs in Schwann cells, sciatic nerve and DRG. a, Quantitative PCR was used to measure the relative levels of mRNAs encoding the subtypes of purinergic receptors in Schwann cells, sciatic nerve and DRG. Data are presented as mean ± SEM; n = 3, PCR reactions for each receptor cDNA were performed in triplicates. **b**, Analysis of purinergic receptor expressions in

Schwann cells, sciatic nerve and DRG by RT-PCR showed that all purinergic receptors mRNAs are expressed. 100 bp DNA ladder was used to indicate the size of the bands. Only one band per gene is visible at the expected band sizes. NT, non-template control.

Analyses of cultured Schwann cells established a pattern of expression with mRNA for three predominant purinergic receptors P2X4, P2Y2 and P2Y13. Transcripts of five purinergic receptors P2Y2, P2Y13, P2Y14, A1, and A2a were dominant in sciatic nerve tissue. In DRG, transcripts of four purinergic receptors P2X3, P2Y2, A1 and A2a were predominant.

Among P2X receptors, the order of the amount of transcripts in cultured Schwann cells was: P2X4 > P2X5 > P2X7 > P2X3 > P2X6 > P2X2 > P2X1. For the sciatic nerve, the order of the amount of transcripts was: P2X4 > P2X5 > P2X6 > P2X1 > P2X2 > P2X3 > P2X7. For DRG, the order of the amount of transcripts was: P2X3 > P2X5 > P2X6 > P2X4 > P2X2 > P2X7 > P2X1.

Among P2Y receptors, the order of the amount of transcripts in cultured Schwann cells was: P2Y13 > P2Y2 > P2Y12 > P2Y14 > P2Y4 > P2Y1 > P2Y6. For the sciatic nerve, the order of the amount of transcripts was: P2Y2 > P2Y13 > P2Y14 > P2Y6 > P2Y1 > P2Y12 > P2Y4. For DRG, the order of the amount of transcripts was: P2Y2 > P2Y1 > P2Y14 > P2Y6 > P2Y13 > P2Y12 > P2Y4.

Among P1 receptors, the order of the amount of transcripts in cultured Schwann cells was: A2a > A2b > A3 > A1. For the sciatic nerve, the order of the amount of transcripts was: A1 > A2a > A2b > A3. For DRG, the order of the amount of transcripts was: A1 > A2a > A2b > A3.

5.4.2 Expression of purinergic receptor proteins in sciatic nerve and DRG

The immunohistochemistry method was used to observe the expression and celluar distribution of purinergic receptors (except P2Y11 receptor) in protein levels in the sciatic nerve and DRG. It was shown that the antibodies for the 18 purinergic receptor subtypes labelled in nerve fibres/neurons and Schwann cells/satellite cells in different intensities (Figure 5-2 - 5-19). The results from our immunohistochemical study indicated that DRG neurons and the sciatic nerve express a variety of purinergic receptor subtypes. Differences in the level of expression of each receptor exist in different cell types of DRG neurons and the sciatic nerve, which shows the diversity of receptor subtypes involved in the physiological function of the DRG and sciatic nerve.

P2X1

The P2X1 receptor subtype was strongly expressed in DRG. Its expression was evenly distributed in the neurons of different sizes. The P2X1 receptors were not expressed in the satellite cells. In the sciatic nerve, the P2X1 receptor subtype was expressed in the nerve fibre as well as on Schwann cells. Intensities of immunoreactivity of P2X1 receptor on nerve fibre were similar to that on Schwann cells (moderate level, Figure 5-2).

P2X2

The P2X2 receptor subtype was expressed moderately in DRG, and was also expressed equally in the neurons of different sizes. However, it was not detected on satellite cells. In the sciatic nerve, P2X2 immunoreactivity was detected in both nerve fibres and Schwann cells at moderate level (Figure 5-3).

P2X3

P2X3 receptor expression pattern in DRG was notable. Small to medium size neurons had very strong P2X3 receptor expression, while very few large size neurons were P2X3 positive. Additionally, even for small and medium size neurons, not all neurons expressed strong P2X3 immunoreactivity. Nearly half of them were almost P2X3 negative. On the other hand, satellite cells around large size neurons were P2X3 positive (with a moderate level of immunoreactivity). In the sciatic nerve, P2X3 receptor immunoreactivity was detected in the nerve fibre as well as on Schwann cells at moderate level (Figure 5-4).

P2X4

In DRG, P2X4 receptor expression was strong in neurons of all sizes. For satellite cells, P2X4 receptor subtype was expressed at moderate immunoreactivity intensities. In the sciatic nerve, the nerve fibre and Schwann cells both expressed P2X4 receptors at moderate level (Figure 5-5).

P2X5

In DRG, P2X5 receptors were expressed moderately in neurons of all sizes. There were some signal intensity variances but it was size-independent. For satellite cells, only very few cells expressed P2X5 receptors at very low (just detectable) levels. In the sciatic nerve, P2X5 receptor expression was moderate in some nerve fibres. Many more Schwann cells expressed P2X5 receptors at similar moderate level (Figure 5-6).

P2X6

In DRG, P2X6 receptor subtype was expressed evenly in neurons of all sizes and its expression level was strong. Some satellites cells also expressed P2X6 receptors but their

expression was lower than that of DRG neurons (i.e. just detectable). In the sciatic nerve, P2X6 receptor expression was moderate in nerve fibres and Schwann cells (Figure 5-7).

P2X7

In DRG, the P2X7 receptor subtype was expressed in neurons of all sizes at just detectable level. Additionally, P2X7 receptors were expressed widely on satellite cells with strong immunoreactivity. In the sciatic nerve, P2X7 receptor immunoreactivity was just detectable in nerve fibres while a strong signal was observed in Schwann cells, which is consistent with the immunoreactivity patterns in DRG neurons and satellite cells (Figure 5-8).

Among the P2X receptors, the order of immunoreactivity intensities in DRG neurons was: P2X3 > P2X1, P2X4, P2X6 > P2X2, P2X5 > P2X7. Among the five P2X receptors which were expressed in satellite cells, the order of immunoreactivity intensities was: P2X7 > P2X3, P2X4 > P2X5, P2X6. For axons in the sciatic nerve, the order of immunoreactivity intensities was: P2X1, P2X2, P2X3, P2X4, P2X5, P2X6 > P2X7. For Schwann cells in the sciatic nerve, the order of immunoreactivity intensities was: P2X7 > P2X1, P2X2, P2X3, P2X4, P2X5, P2X6.

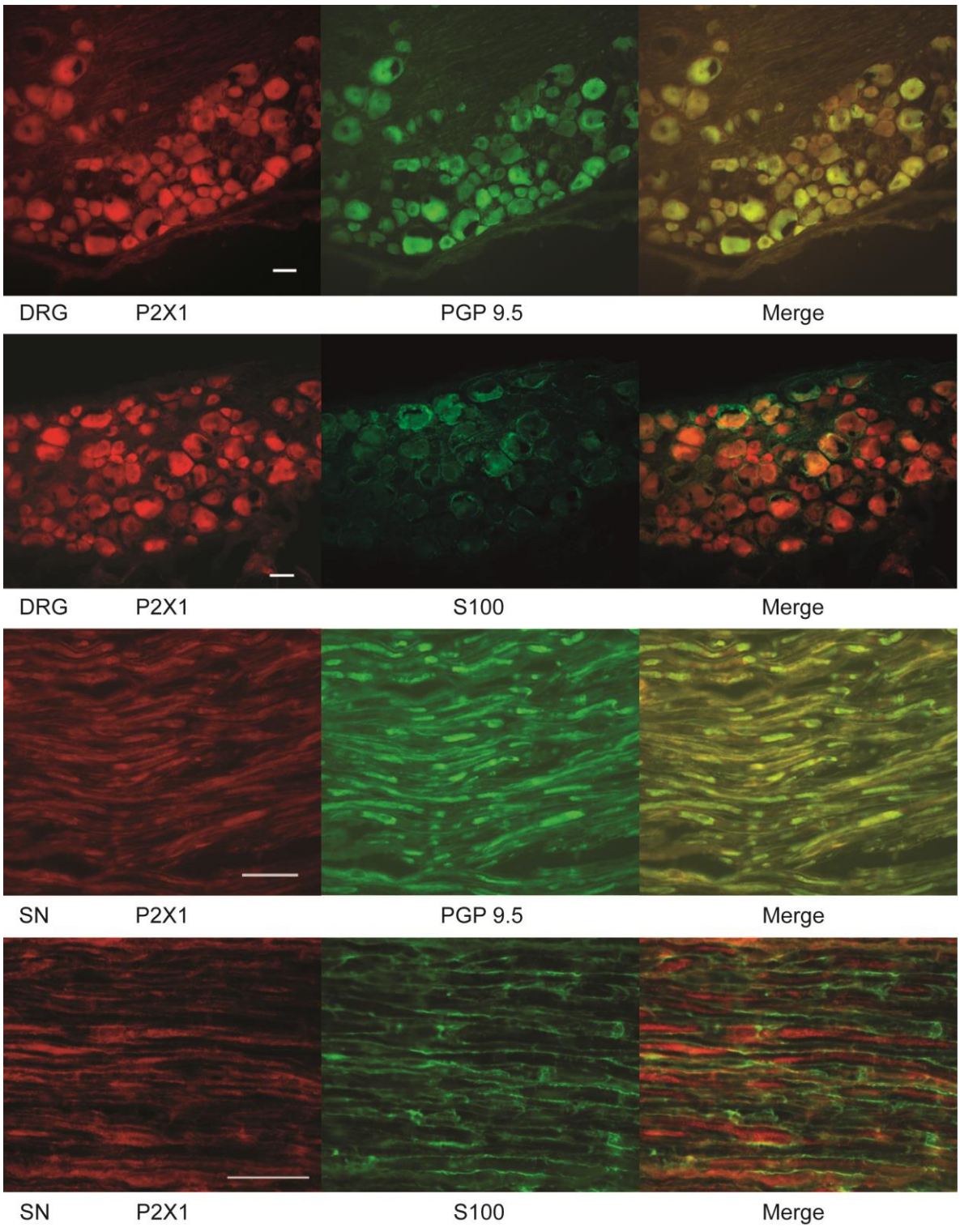

Figure 5-2 Expression of P2X1 receptor in adult rat DRG and sciatic nerve. Co-immunohistochemical labelling of PGP 9.5 or S100. Scale bar, 50 μm.

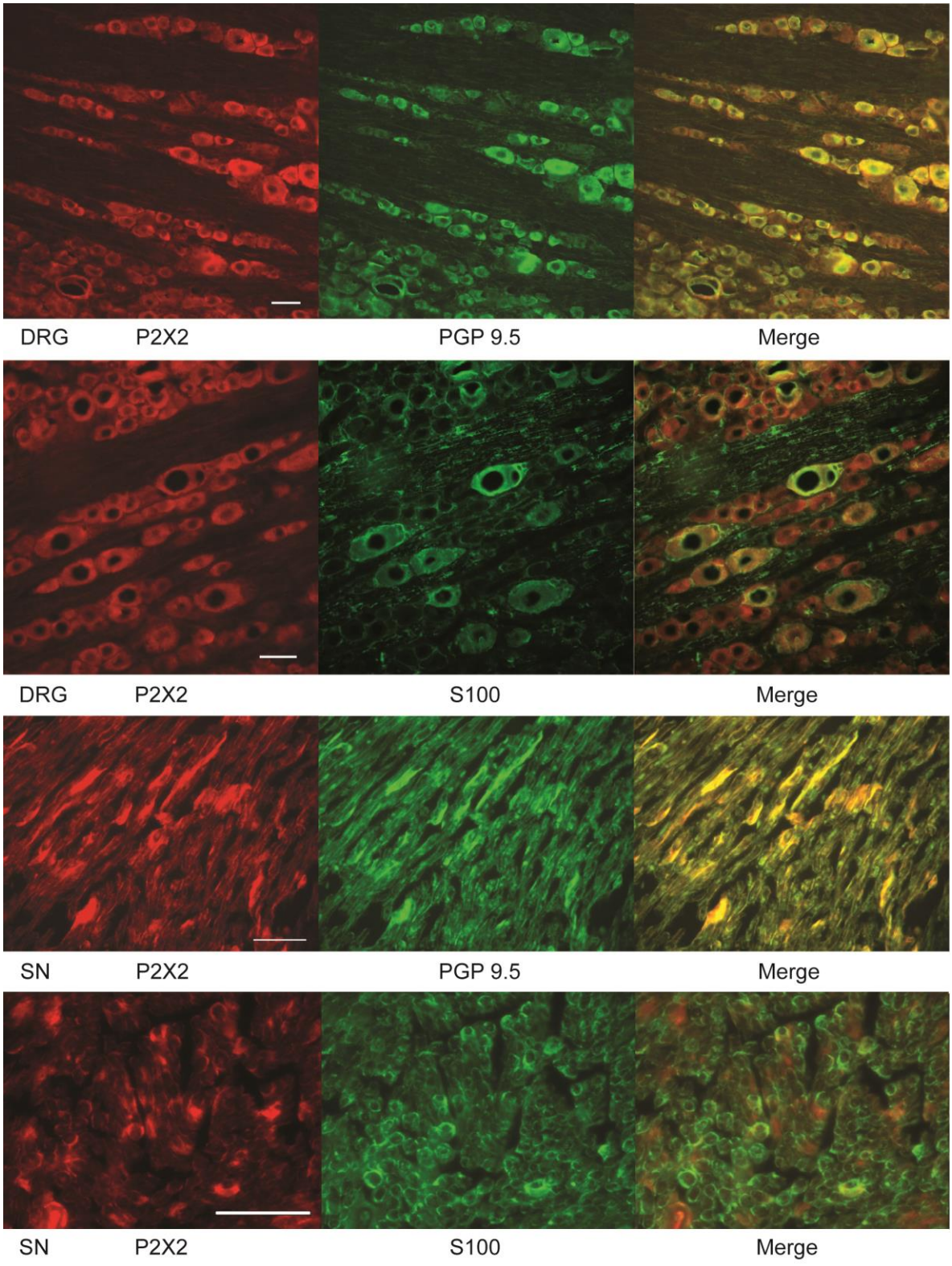

Figure 5-3 Expression of P2X2 receptor in adult rat DRG and sciatic nerve. Co-immunohistochemical labelling of PGP 9.5 or S100. Scale bar, 50 μm.

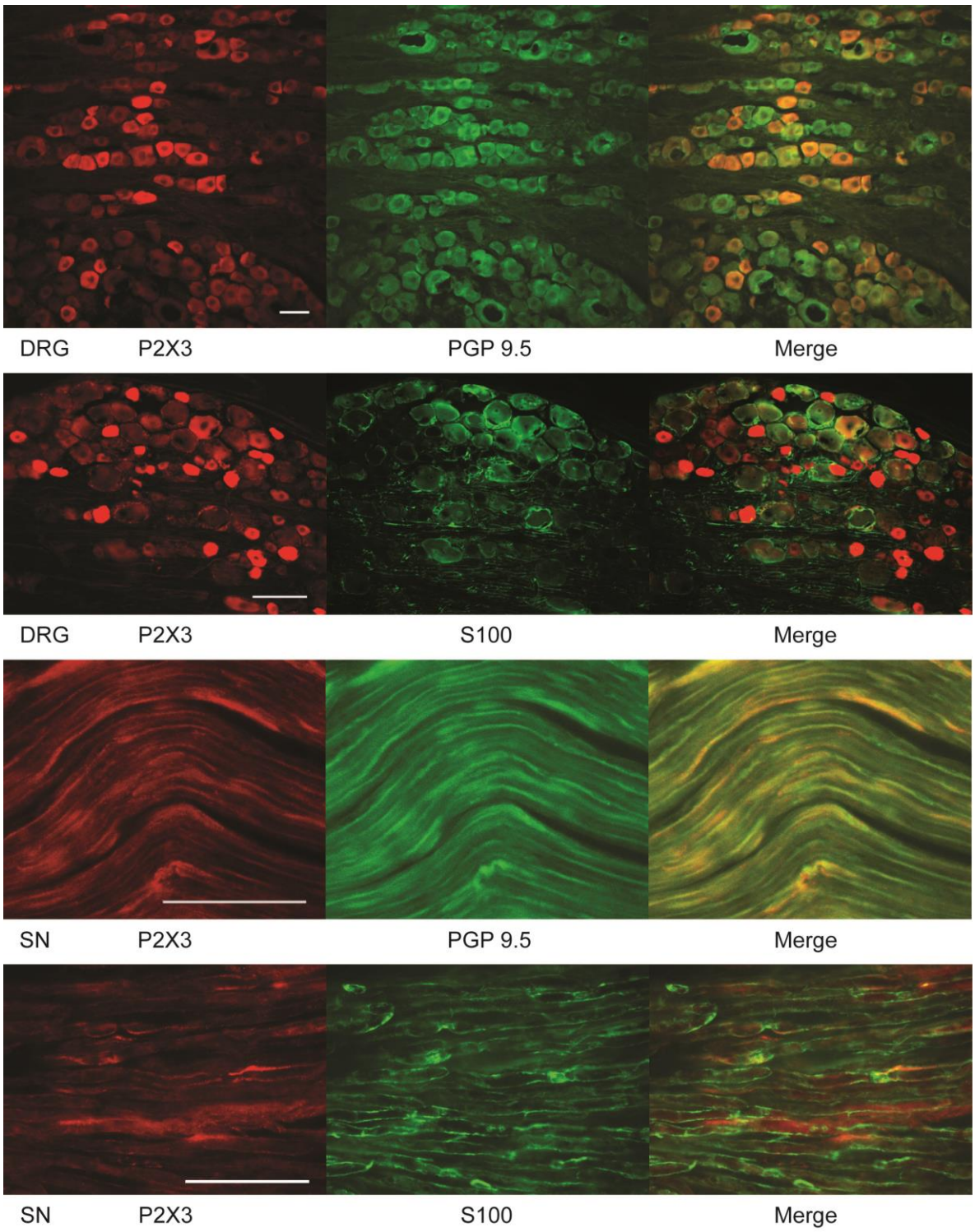

Figure 5-4 Expression of P2X3 receptor in adult rat DRG and sciatic nerve. Co-immunohistochemical labelling of PGP 9.5 or S100. Scale bar, 50 μm.

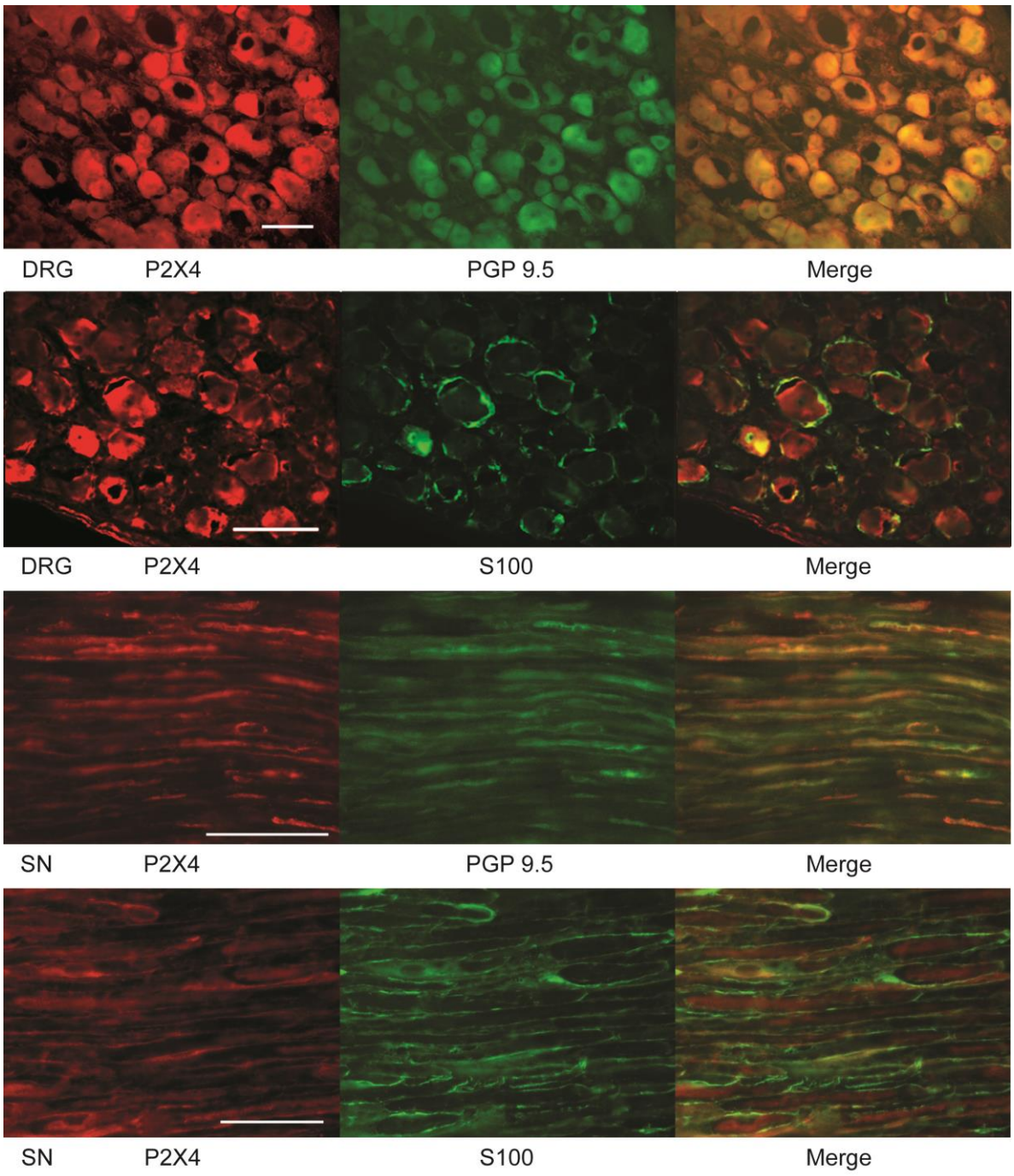

Figure 5-5 Expression of P2X4 receptor in adult rat DRG and sciatic nerve. Co-immunohistochemical labelling of PGP 9.5 or S100. Scale bar, 50 μm.

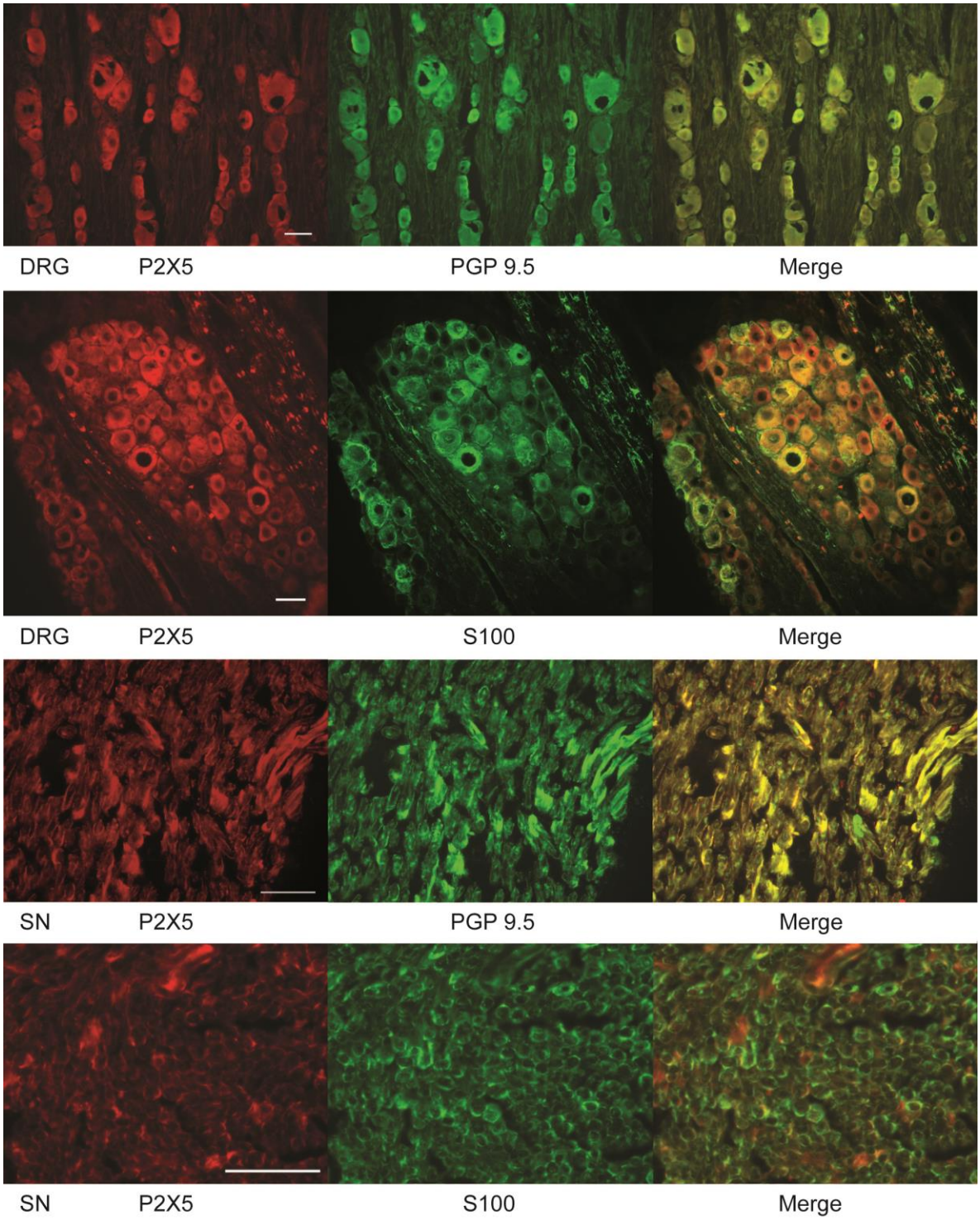

Figure 5-6 Expression of P2X5 receptor in adult rat DRG and sciatic nerve. Co-immunohistochemical labelling of PGP 9.5 or S100. Scale bar, 50 μm.

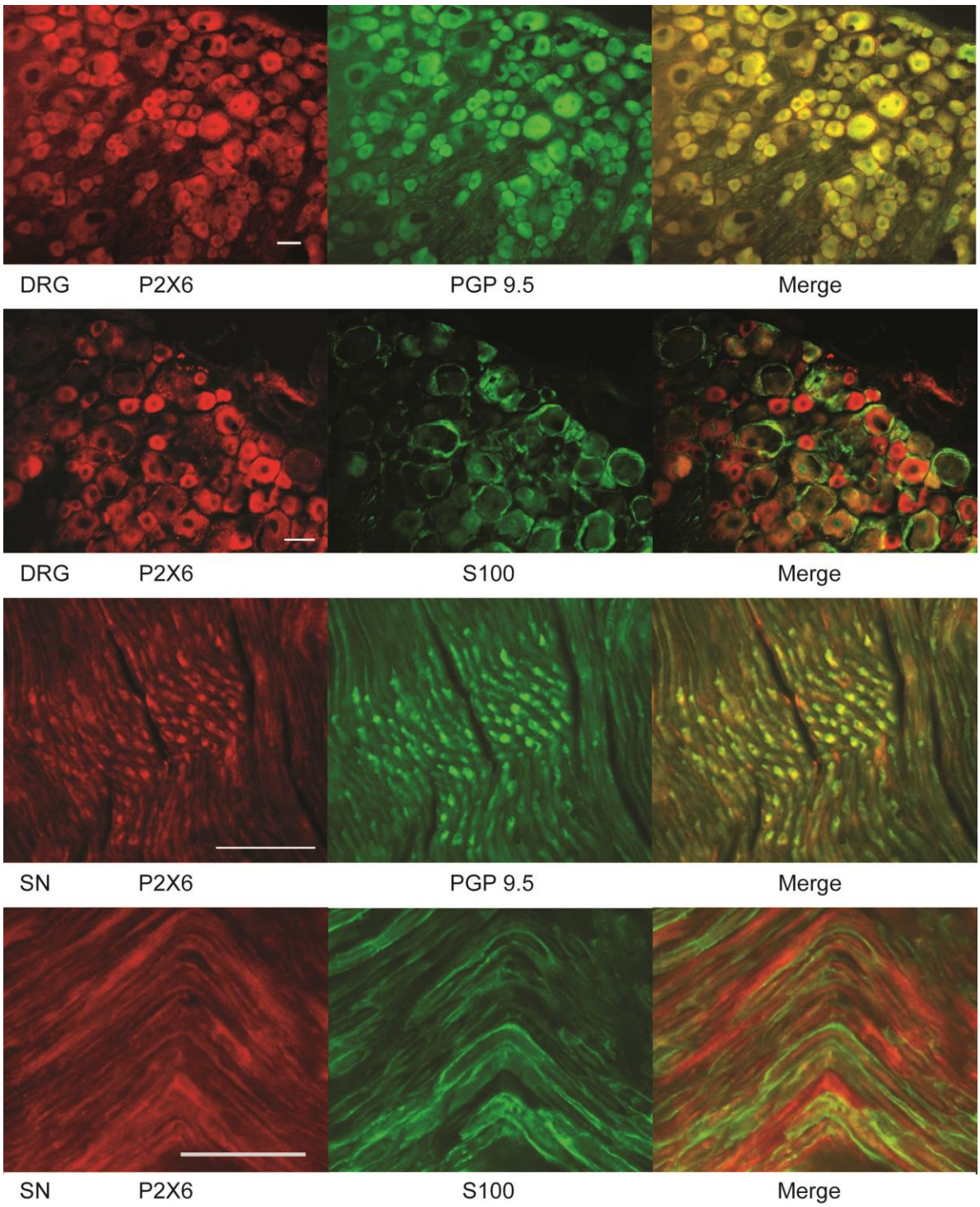

Figure 5-7 Expression of P2X6 receptor in adult rat DRG and sciatic nerve. Co-immunohistochemical labelling of PGP 9.5 or S100. Scale bar, 50 μm.

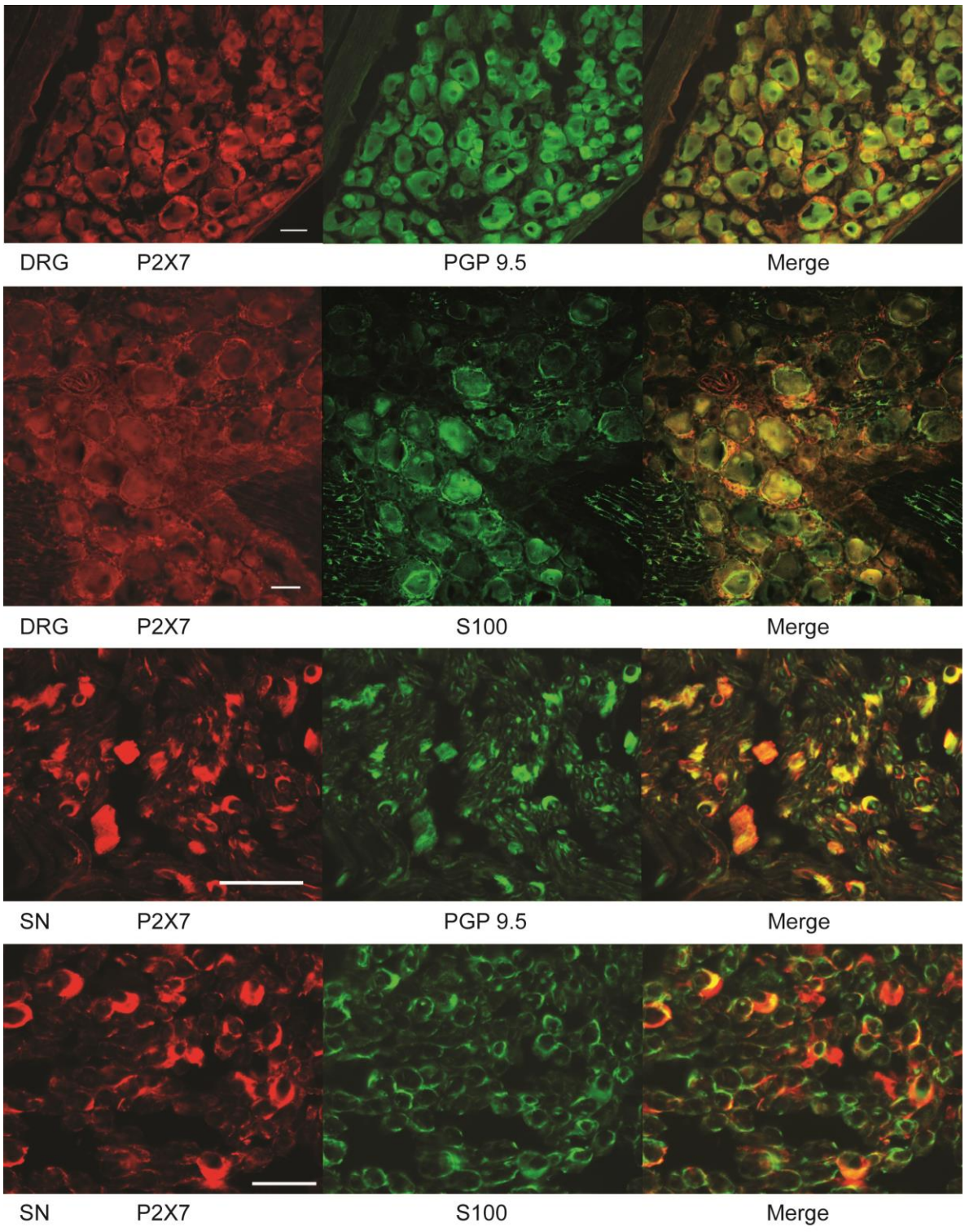

Figure 5-8 Expression of P2X7 receptor in adult rat DRG and sciatic nerve. Co-immunohistochemical labelling of PGP 9.5 or S100. Scale bar, 50 μm.

P2Y1

In DRG, the P2Y1 receptor subtype was expressed in moderate levels in neurons of all sizes. In particular, the P2Y1 immunoreactivity was also detected in the nuclei of neurons at a moderate level. P2Y1 receptors were not detected in satellite cells. In the sciatic nerve, just detectable P2Y1 immunoreactivity was observed in nerve fibres and moderate immunoreactivity was detected in Schwann cells (Figure 5-9).

P2Y2

In DRG, a moderate P2Y2 receptor immunoreactivity was detected in neurons of all sizes. Interestingly, strong P2Y2 receptor expression was observed in nearly all satellite cells around the neurons of all sizes. In the sciatic nerve, P2Y2 receptor expression was strong in both nerve fibres and Schwann cells (Figure 5-10).

P2Y4

The P2Y4 receptor subtype was expressed in DRG neurons of all sizes at a just detectable level. Additionally, a few satellite cells expressed P2Y4 receptors at moderate levels. In the sciatic nerve, P2Y4 was detected in both nerve fibres and Schwann cells at moderate levels (Figure 5-11).

P2Y6

The P2Y6 receptor subtype was moderately expressed in DRG neurons of all sizes. Some satellite cells expressed P2Y6 receptors at a similar level to that of DRG neurons. In the sciatic nerve, P2Y6 was detected in some nerve fibres at moderate levels and moderate P2Y6 expression was observed in most of Schwann cells. (Figure 5-12).

P2Y12

P2Y12 receptor expression pattern in DRG was unique. DRG neurons of all sizes expressed a moderate P2Y12 receptor immunoreactivity in their cytoplasm, but strong P2Y12 receptor immunoreactivity was detected in the nuclei of DRG neurons. In addition, some satellite cells expressed moderate P2Y12 receptor immunoreactivity. In the sciatic nerve, nerve fibres expressed P2Y12 receptors at just detectable levels, while Schwann cells expressed strong P2Y12 receptor immunoreactivity (Figure 5-13).

P2Y13

P2Y13 receptor expression of DRG neurons of all sizes was at moderate levels. P2Y13 receptor expression was not observed in satellite cells. In the sciatic nerve, some axons expressed P2Y13 at just detectable levels while Schwann cells expressed P2Y13 at a very strong level. (Figure 5-14).

P2Y14

The P2Y14 receptor subtype was expressed in low (just detectable) levels in DRG neurons of all sizes. It was noteworthy that P2Y14 receptor expression was also observed in the nuclei of some neurons at low levels. P2Y14 receptors were not detected in satellite cells. In the sciatic nerve, the P2Y14 receptor subtype was detected at similarly moderate levels in both nerve fibres and Schwann cells (Figure 5-15).

In P2X7, P2Y2, P2Y4, P2Y12, P2Y13 and P2Y14 receptors immunostaining, there were strong, distinctive immunoreactivities in Schwann cells. This is mainly due to the high level localization of these receptors on Schmidt-Lanterman incisures (myelin incisures), conical tube-like cytoplasmic structures that cross the compact myelin and connect the

peripheral and the periaxonal cytoplasm of Schwann cells (Tricaud et al., 2005). It is suggested that these incisures are important for the transport of metabolic substances across the myelin sheath and for the maintenance and longitudinal growth of the sheath (Ghabriel and Allt, 1981, Arroyo and Scherer, 2000).

Among P2Y receptors, the order of immunoreactivity intensities in DRG neurons was: P2Y1, P2Y2, P2Y6, P2Y12, P2Y13 > P2Y4, P2Y14. Among the four P2Y receptors which were expressed in satellite cells, the order of immunoreactivity intensities was P2Y2 > P2Y4, P2Y6, P2Y12. For axons in the sciatic nerve, the order of immunoreactivity intensities was: P2Y2 > P2Y4, P2Y6, P2Y14 > P2Y1, P2Y12, P2Y13. For Schwann cells in the sciatic nerve, the order of immunoreactivity intensities was: P2Y13 > P2Y2, P2Y12 > P2Y1, P2Y4, P2Y6, P2Y14.

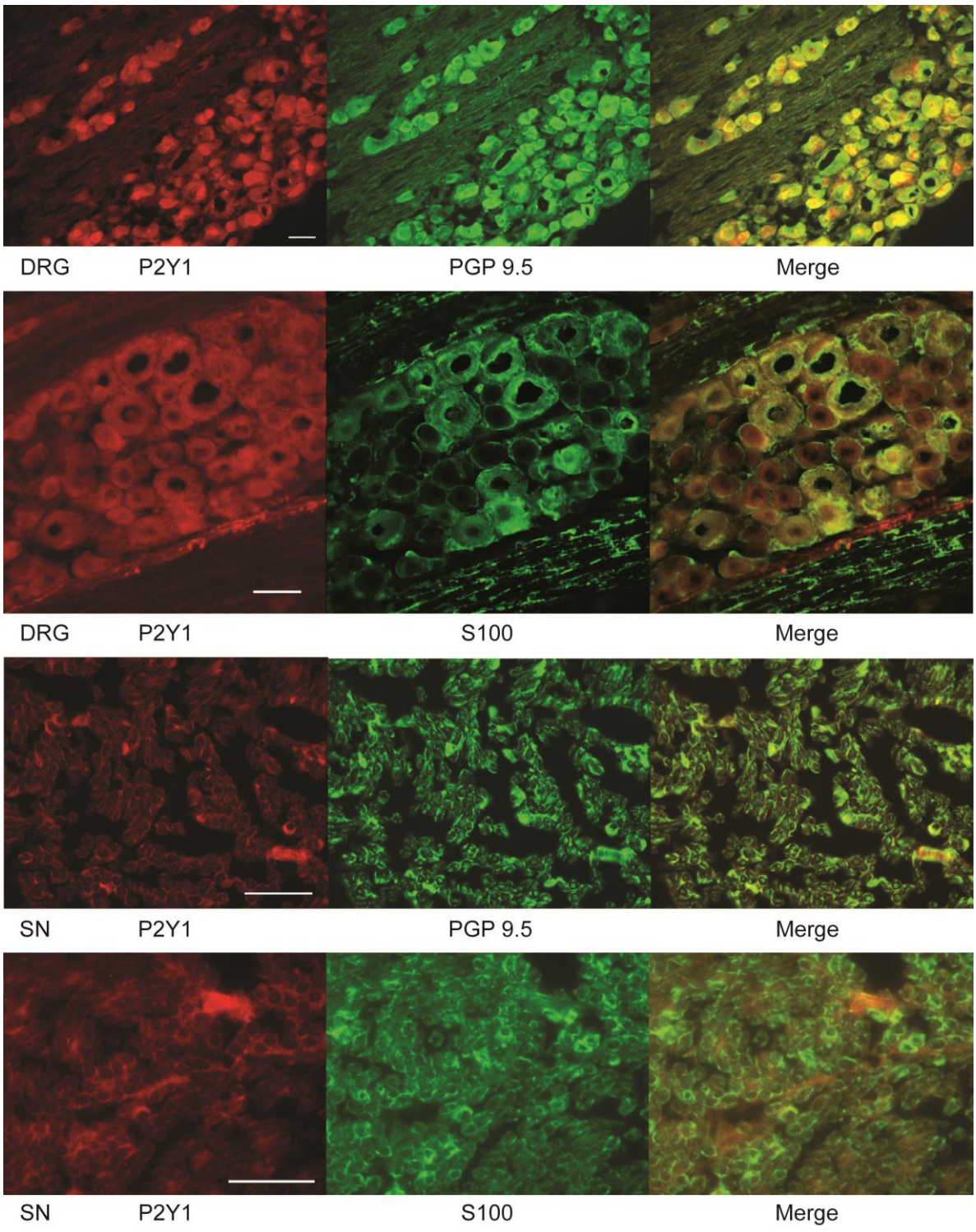

Figure 5-9 Expression of P2Y1 receptor in adult rat DRG and sciatic nerve. Co-immunohistochemical labelling of PGP 9.5 or S100. Scale bar, 50 μm.

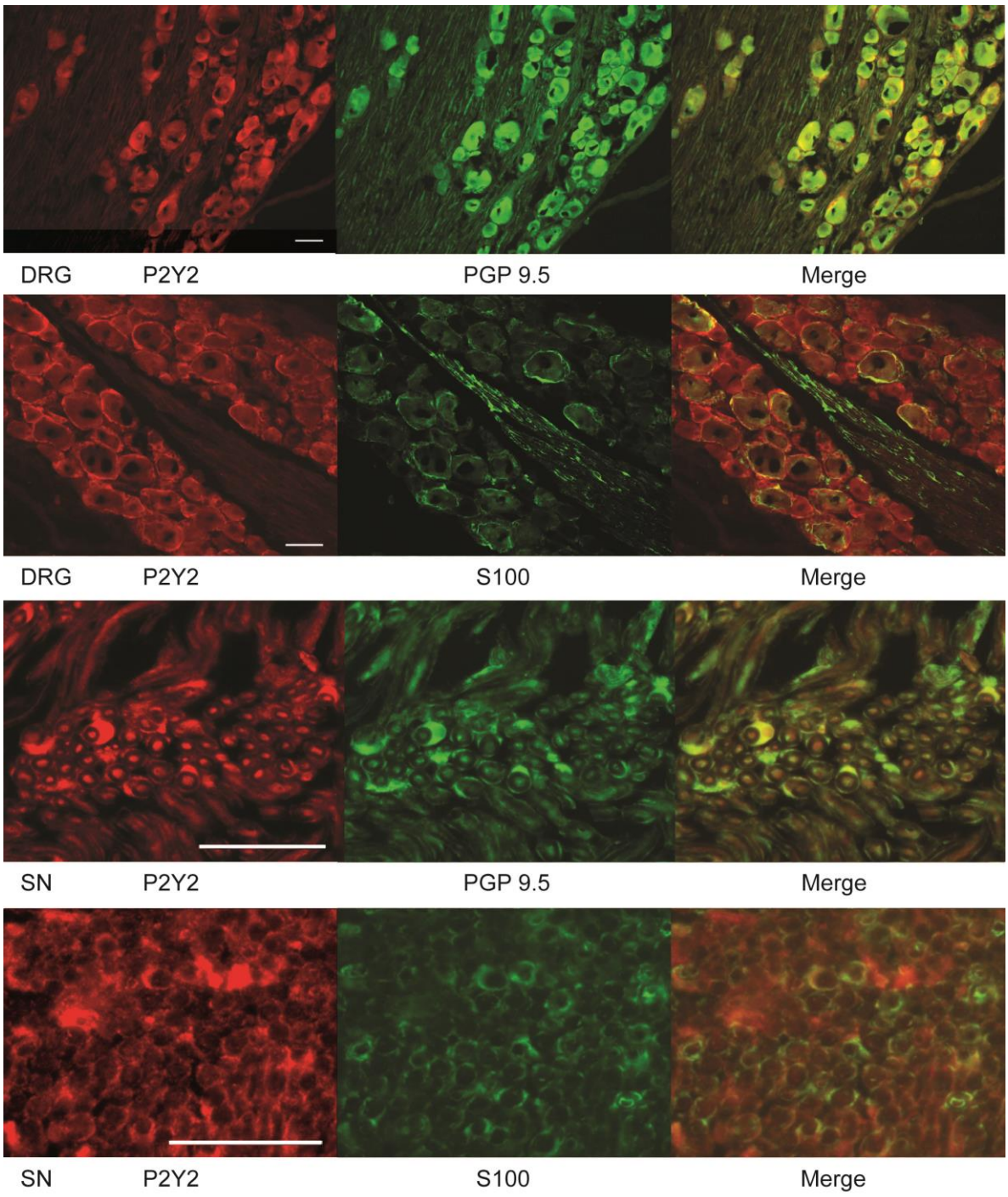

Figure 5-10 Expression of P2Y2 receptor in adult rat DRG and sciatic nerve. Co-immunohistochemical labelling of PGP 9.5 or S100. Scale bar, 50 μm.

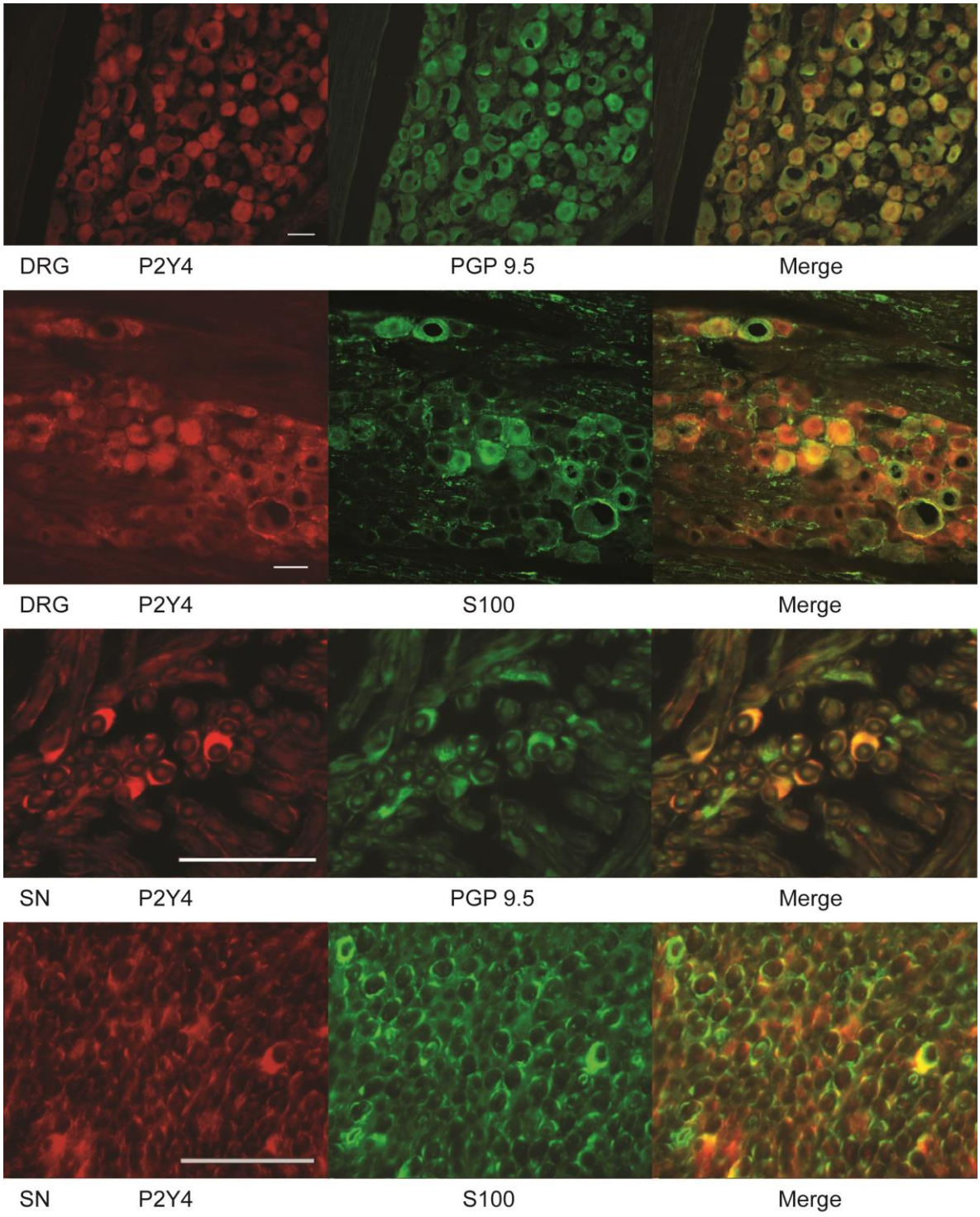

Figure 5-11 Expression of P2Y4 receptor in adult rat DRG and sciatic nerve. Co-immunohistochemical labelling of PGP 9.5 or S100. Scale bar, 50 µm.

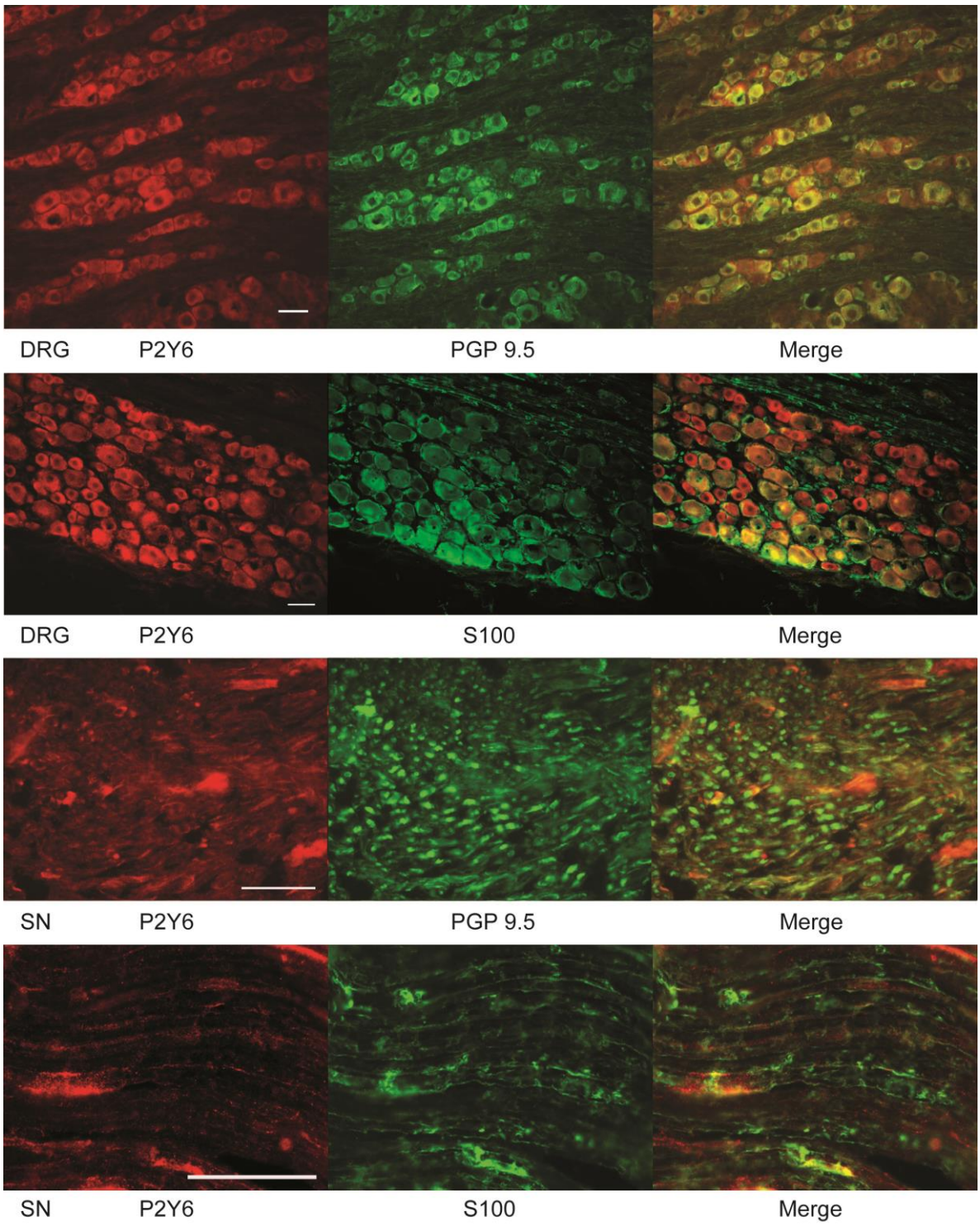

Figure 5-12 Expression of P2Y6 receptor in adult rat DRG and sciatic nerve. Co-immunohistochemical labelling of PGP 9.5 or S100. Scale bar, 50 µm.

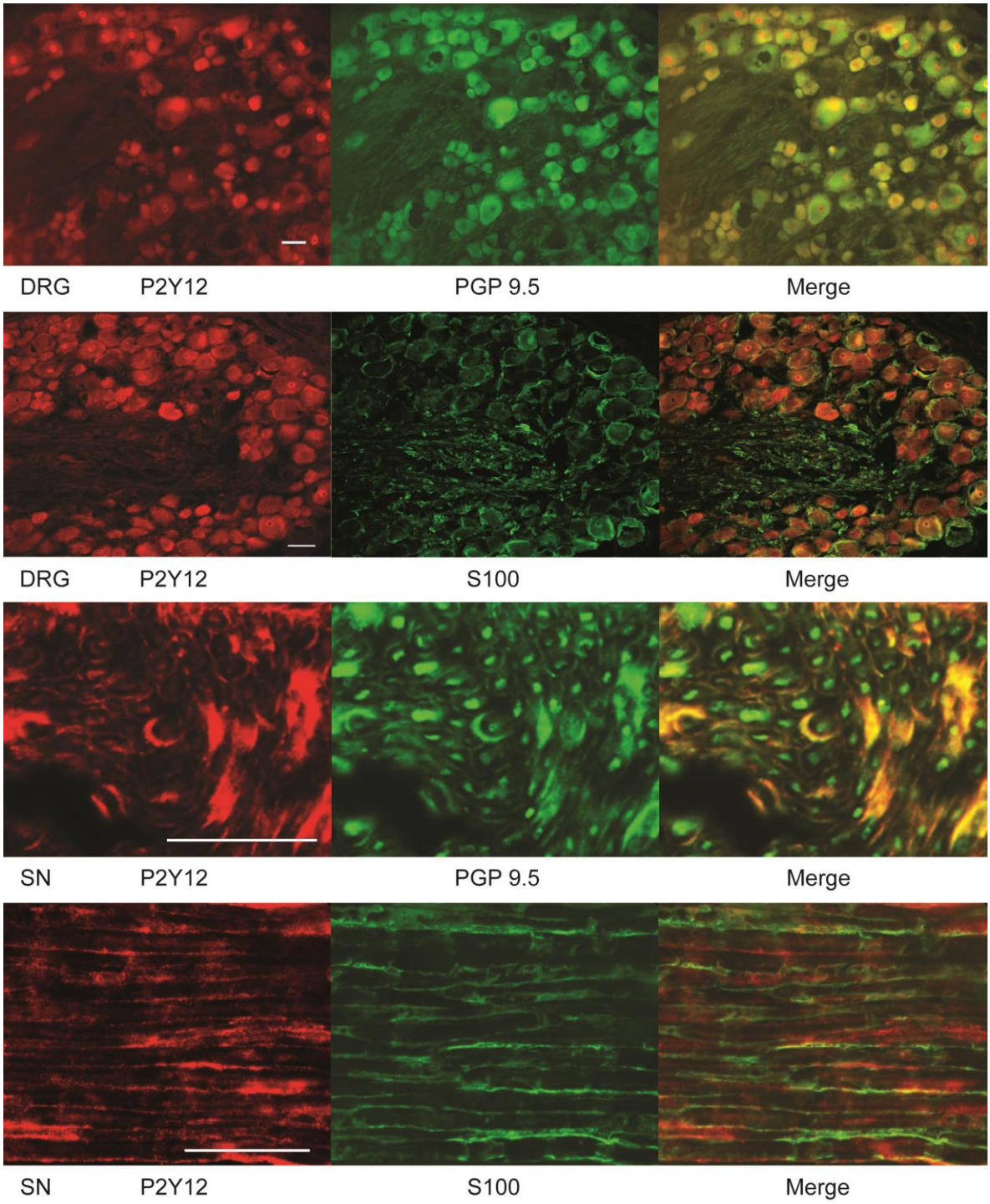

Figure 5-13 Expression of P2Y12 receptor in adult rat DRG and sciatic nerve. Co-immunohistochemical labelling of PGP 9.5 or S100. Scale bar, 50 µm.

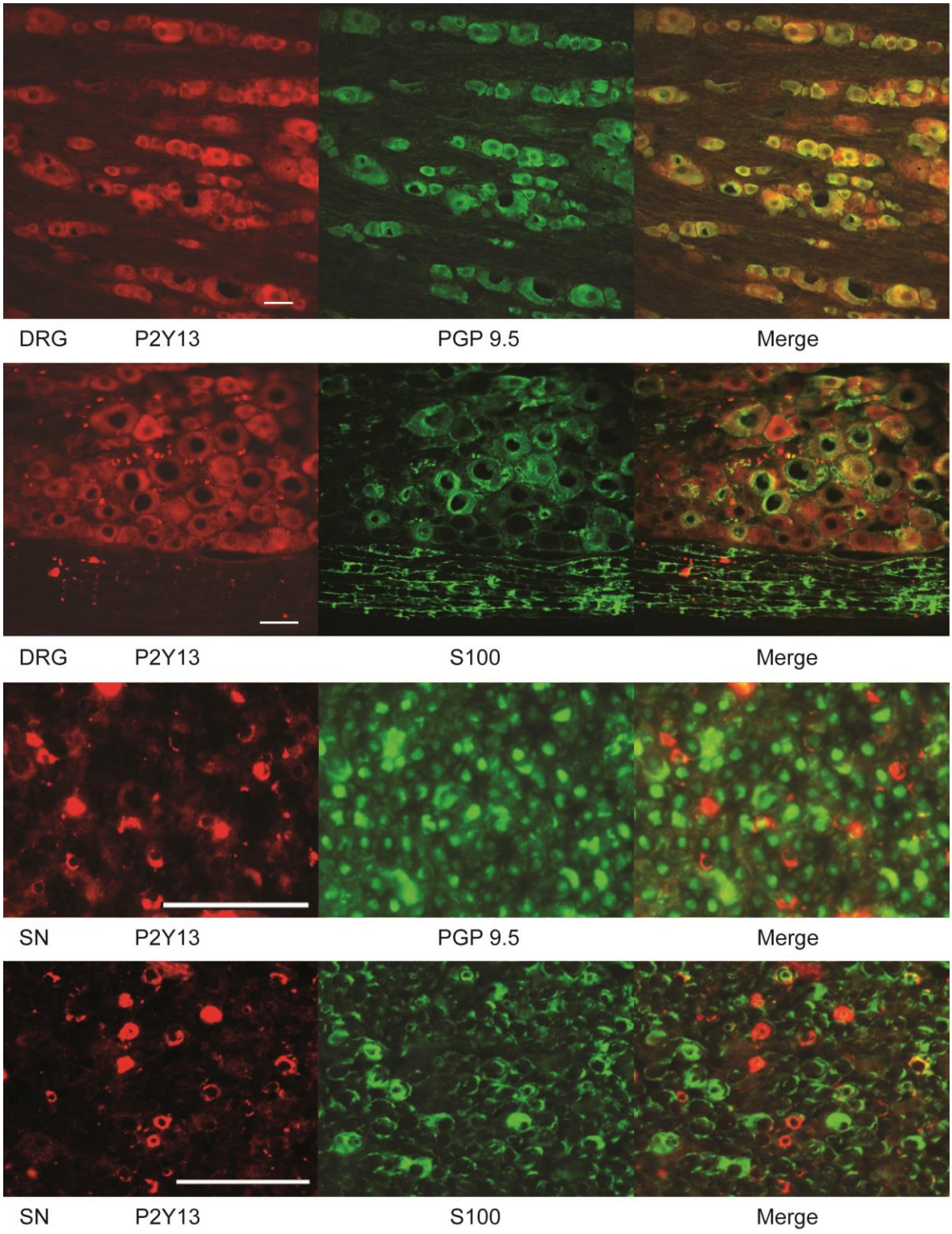

Figure 5-14 Expression of P2Y13 receptor in adult rat DRG and sciatic nerve. Co-immunohistochemical labelling of PGP 9.5 or S100. Scale bar, 50 µm.

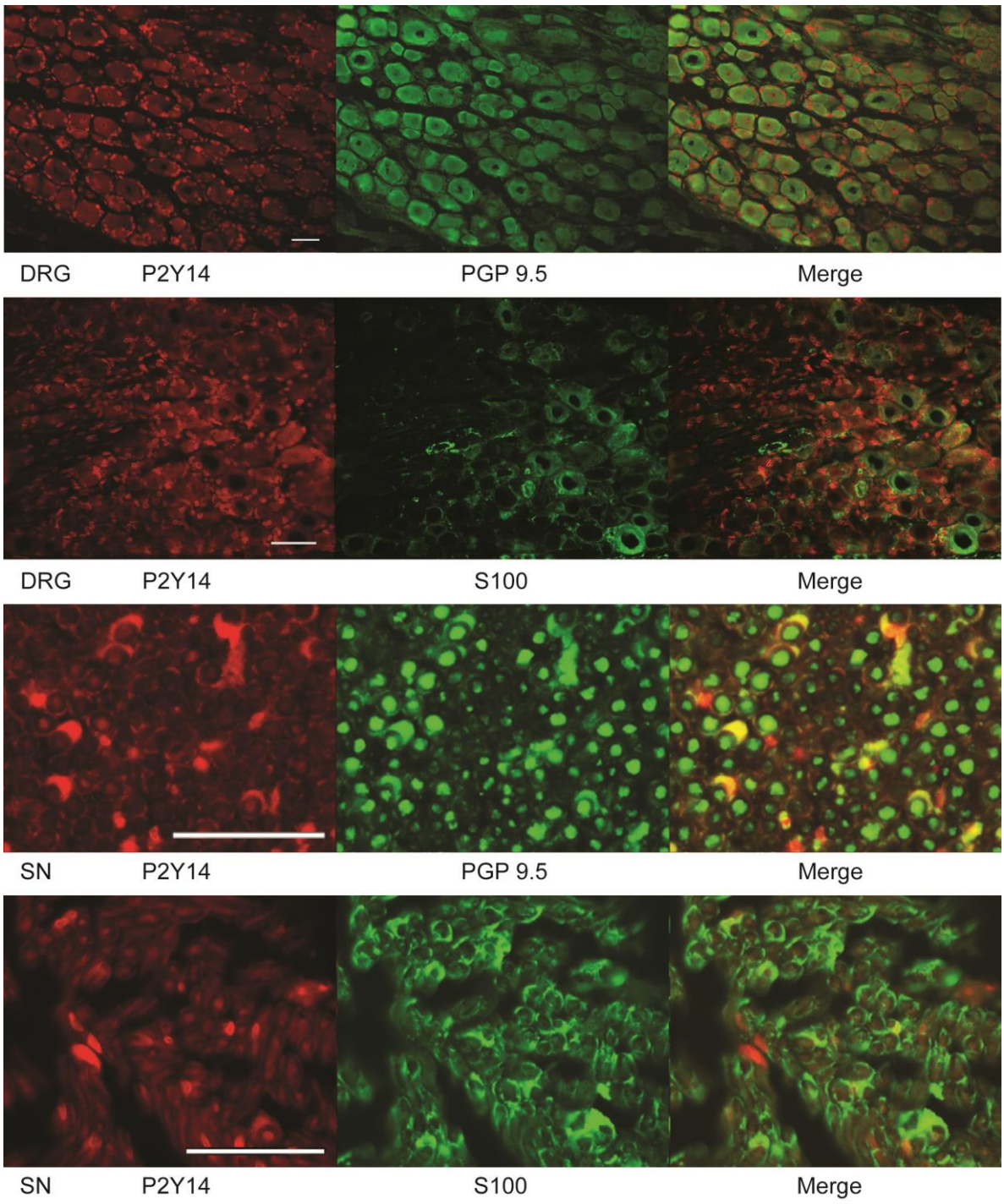

Figure 5-15 Expression of P2Y14 receptor in adult rat DRG and sciatic nerve. Co-immunohistochemical labelling of PGP 9.5 or S100. Scale bar, 50 µm.

A1

Al receptor expression was strong in DRG neurons of all sizes. Few satellite cells expressed A1 receptors at moderate levels. In the sciatic nerve, nerve fibres expressed A1 receptors at moderate levels. A1 receptor immunoreactivity was also detected in Schwann cells at a moderate level (Figure 5-16).

A2a

A2a receptor subtype was detected at moderate levels in DRG neurons of all sizes. A few satellite cells expressed moderate levels of A2a receptors. In the sciatic nerve, both nerve fibres and Schwann cells expressed A2a receptors at a moderate level (Figure 5-17).

A2b

A2b receptor expression was moderate in DRG neurons of all sizes. Only a few satellite cells expressed moderate A2b receptor immunoreactivity. In sciatic nerve, A2b receptor immunoreactivity was just detectable in nerve fibres but expressed at a moderate level in Schwann cells (Figure 5-18).

A3

A3 receptor subtype expression was just detectable in DRG neurons of all sizes. However, no satellite cells expressed A3 receptors. A3 receptor immunoreactivity was just detectable in both nerve fibres and Schwann cells in the sciatic nerve (Figure 5-19).

Among P1 adenosine receptors, the order of immunoreactivity intensities in DRG neurons was: A1 > A2a, A2b > A3. Among the three P1 receptors A1, A2a and A2b, which were expressed in satellite cells, the immunoreactivity intensities were similar. Among P1

receptors, the order of immunoreactivity intensities in the sciatic nerve fibres was: A1, A2a > A2b, A3. For Schwann cells in the sciatic nerve, the order of immunoreactivity intensities was: A1, A2a, A2b > A3.

Immunoreactivity intensities of all types of purinergic receptors in rat sciatic nerve and DRG are summarized in Table 5-1.

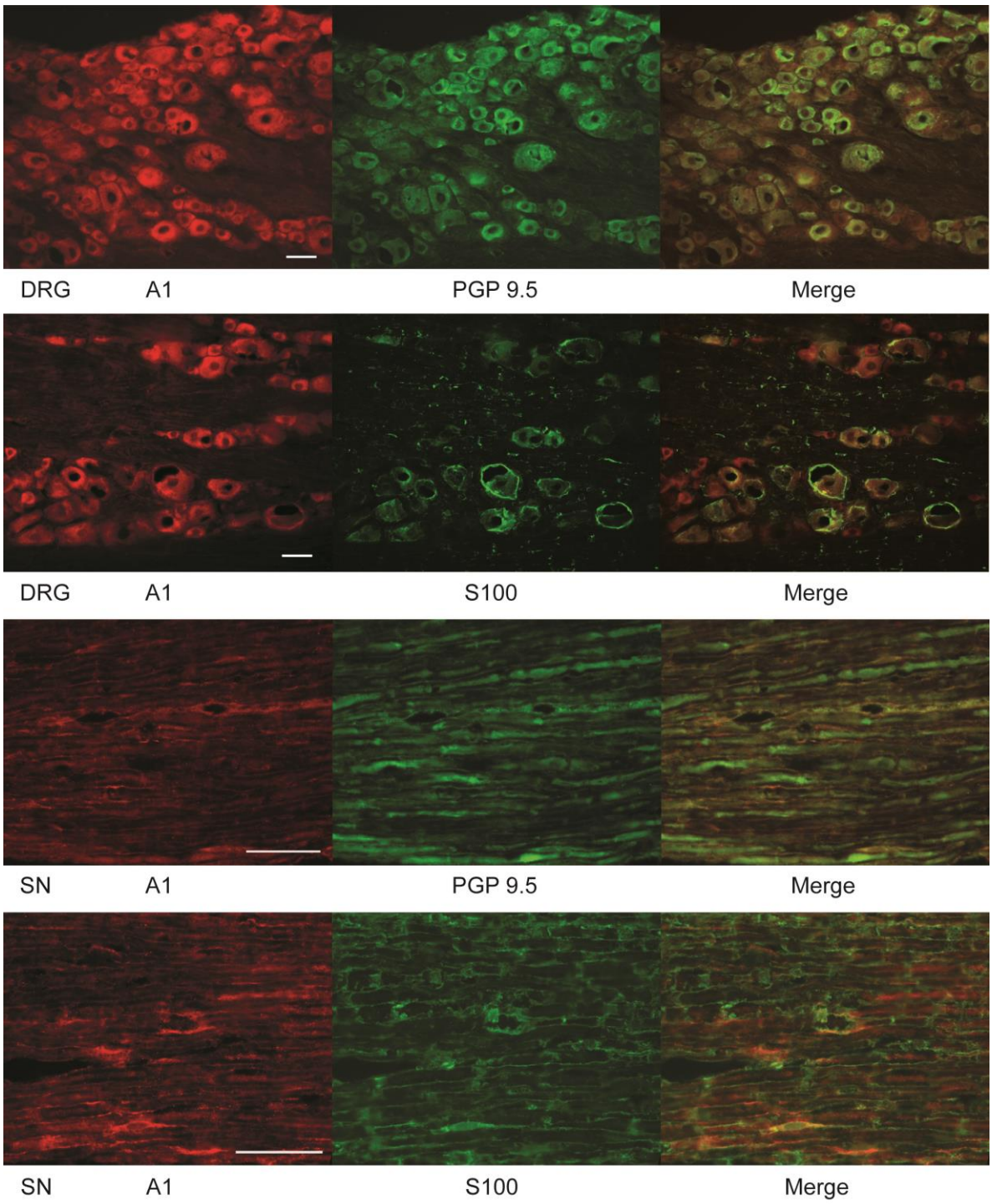

Figure 5-16 Expression of A1 receptor in adult rat DRG and sciatic nerve. Co-immunohistochemical labelling of PGP 9.5 or S100. Scale bar, 50 µm.

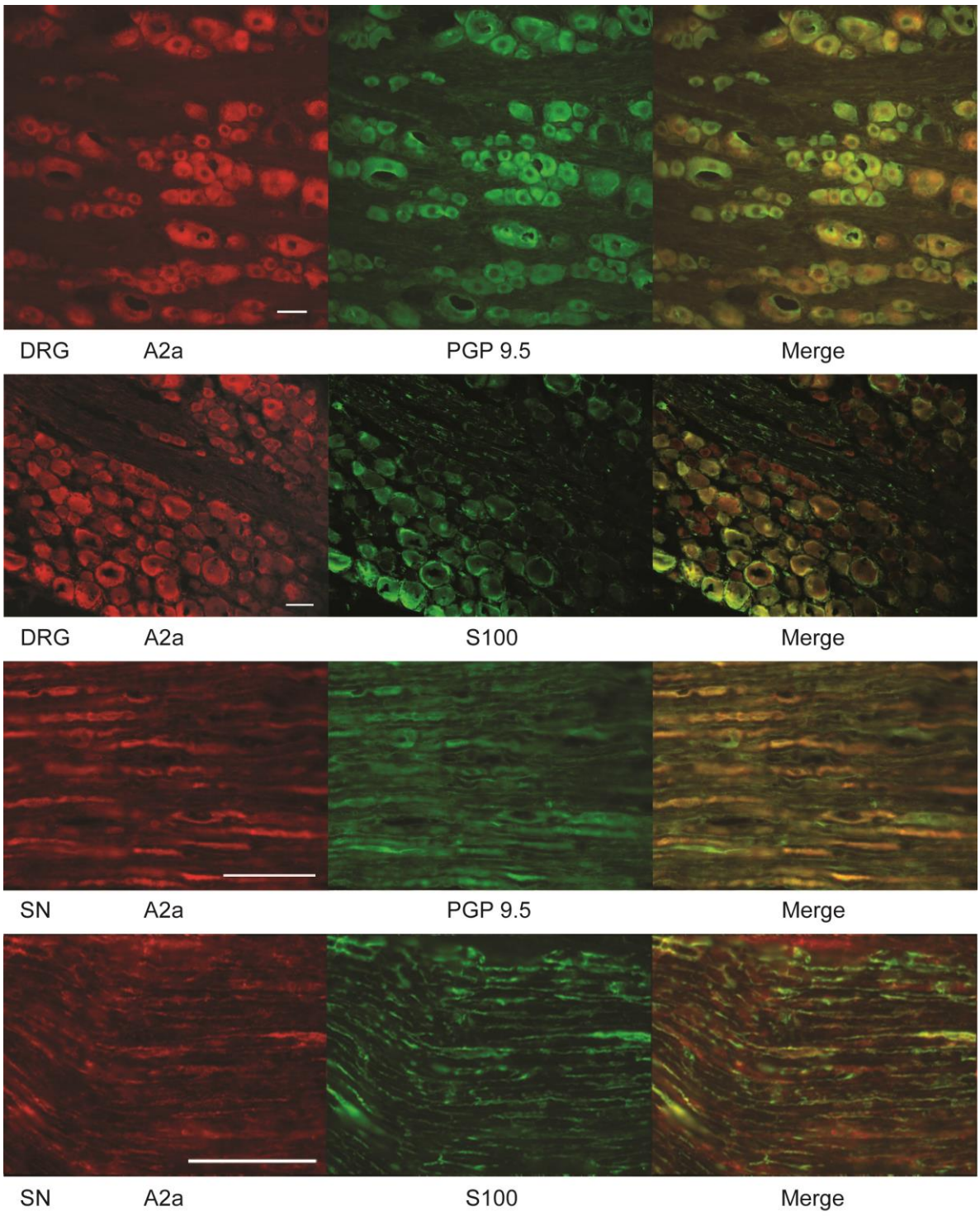

Figure 5-17 Expression of A2a receptor in adult rat DRG and sciatic nerve. Co-immunohistochemical labelling of PGP 9.5 or S100. Scale bar, 50 µm.

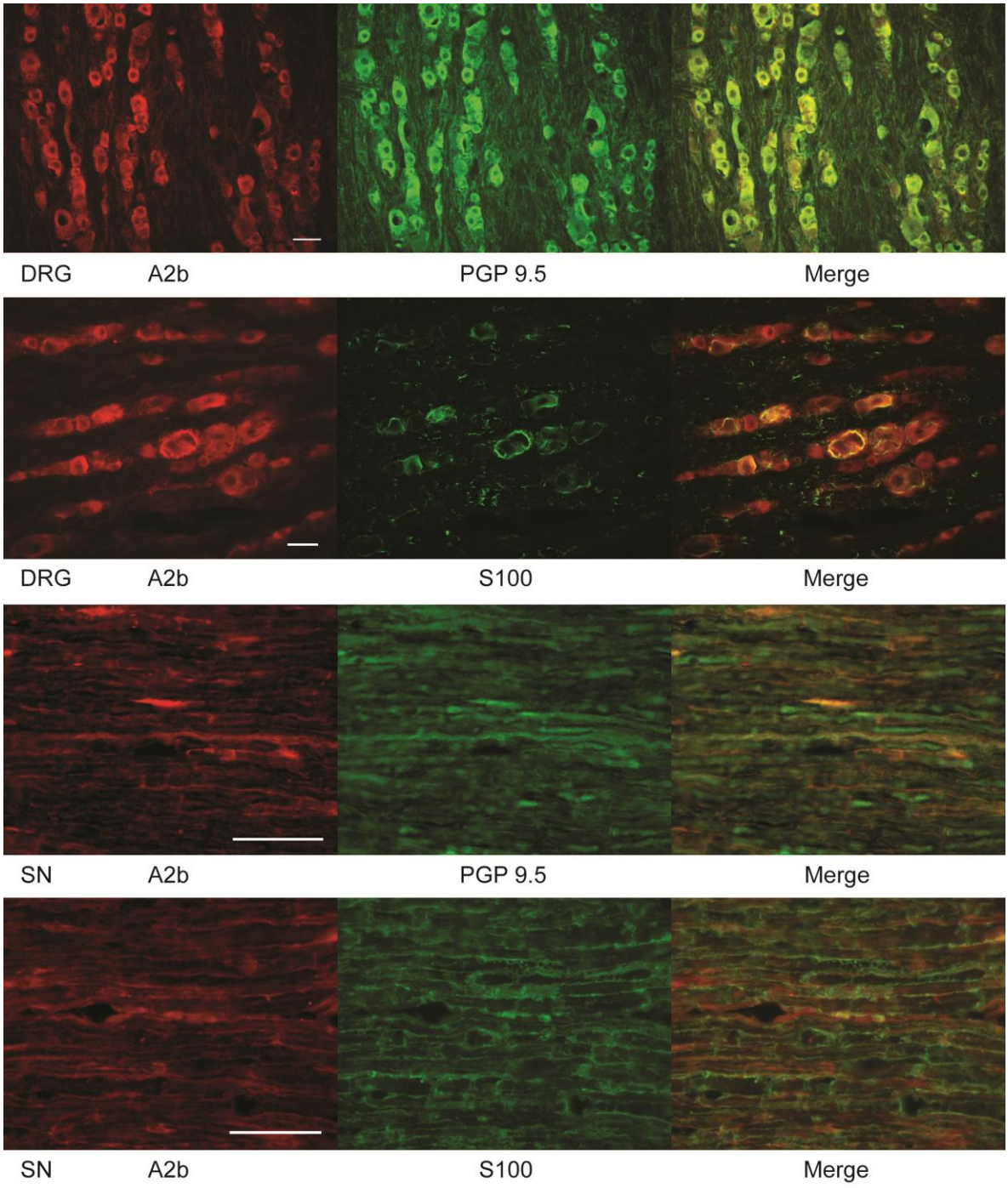

Figure 5-18 Expression of A2b receptor in adult rat DRG and sciatic nerve. Co-immunohistochemical labelling of PGP 9.5 or S100. Scale bar, 50 µm.

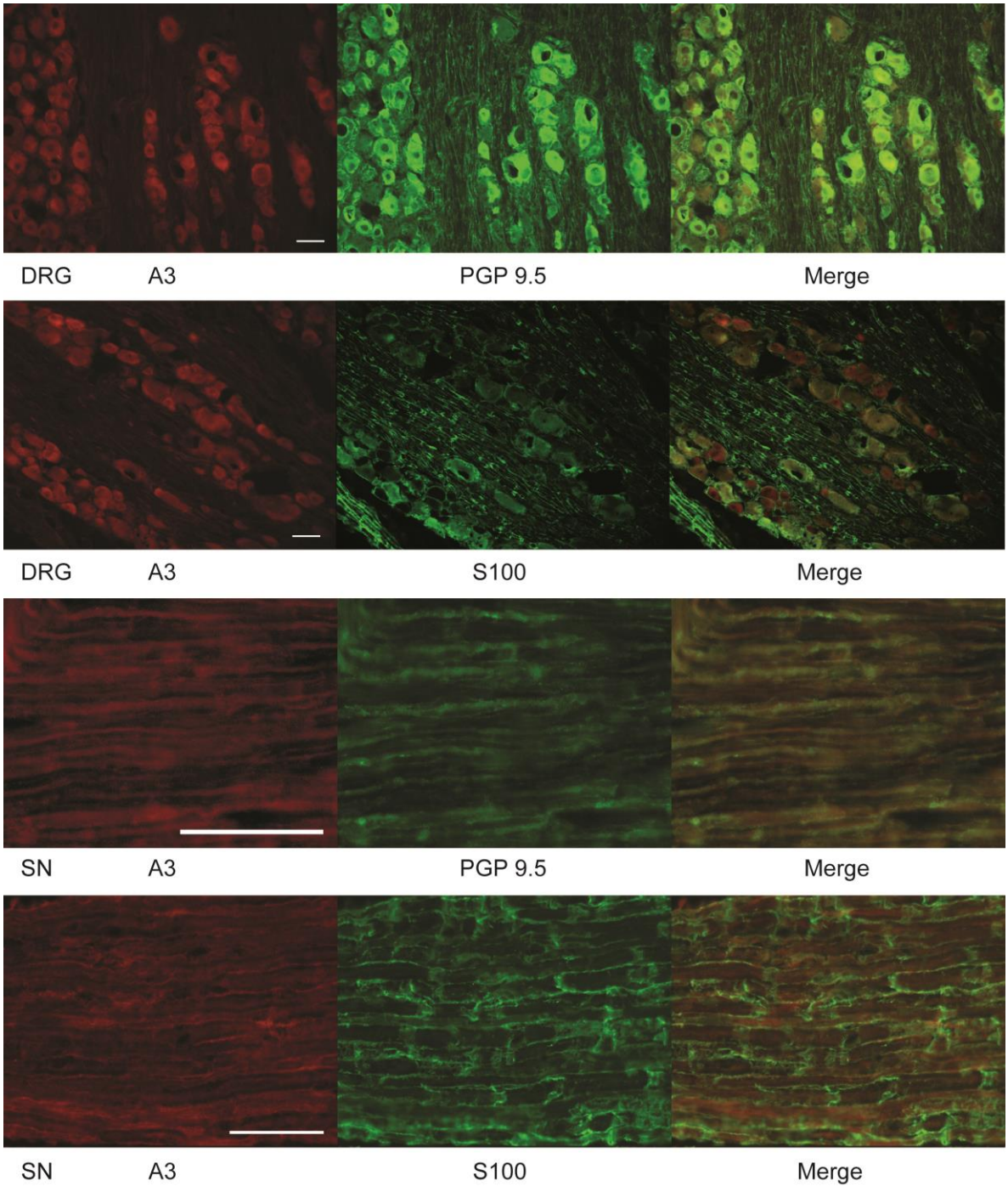

Figure 5-19 Expression of A3 receptor in adult rat DRG and sciatic nerve. Co-immunohistochemical labelling of PGP 9.5 or S100. Scale bar, 50 μm.

Table 5-1 Immunoreactivities of purinergic receptor subtypes in rat sciatic nerve and DRG

	DRG		Sciatic nerve	
Cell type	Neurons (PGP 9.5^{+})	Satellite cells (S100^{+})	Axons (PGP 9.5^{+})	Schwann cells (S100^{+})
P2X1	++	-	+	+
P2X2	+	-	+	+
P2X3	+++*	+	+	+
P2X4	++	+	+	+
P2X5	+	+/-	+	+
P2X6	++	+/-	+	+
P2X7	+/-	++	+/-	++***
P2Y1	+(+**)	-	+/-	+
P2Y2	+	++	++	++***
P2Y4	+/-	+	+	+***
P2Y6	+	+	+	+
P2Y11	Not cloned in rats			
P2Y12	+ (++**)	+	+/-	++***
P2Y13	+	-	+/-	+++***
P2Y14	+/- (+/-**)	-	+	+***
A1	++	+	+	+
A2a	+	+	+	+
A2b	+	+	+/-	+
A3	+/-	-	+/-	+/-

+++, very strong signal; ++, strong signal; +, moderate signal; +/-, just detectable; -, undetectable.

* Almost exclusively expressed in small and medium size neurons.

** Signal level in nuclei.

*** Distinctive immunoreactivities in Schmidt-Lanterman incisures were observed.

To check the specificity of antibodies for purinergic receptors, each antibody was preabsorbed with its antigen for 24 hours. As a result, immunostaining of purinergic receptors in DRG was almost abolished (data not shown). In control experiments, no signal was detected when PBS was applied instead of primary antibodies (data not shown). Also, sciatic nerves from wild-type (WT) and P2X7 receptor knockout (KO) mice were immunostained with polyclonal antibodies against the P2X7 receptor. P2X7 receptor immunoreactivity was detected in the sciatic nerve of WT mice, but absent in the sciatic nerve of the P2X7 receptor KO mice (Figure 5-20).

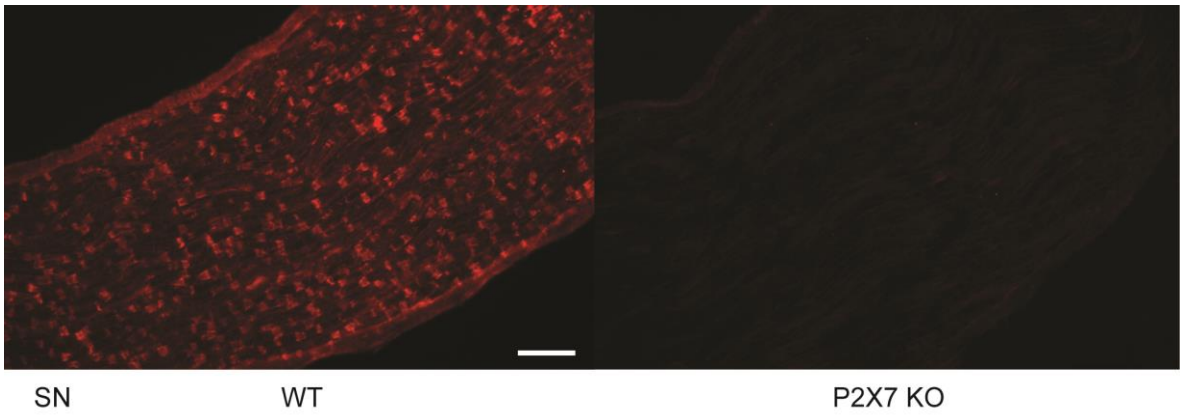

Figure 5-20 Specificity of purinergic receptors antibodies. Sciatic nerve from wild-type (WT) and P2X7 receptor knockout (KO) mice were immunostained for P2X7 receptor. P2X7 KO mice showed no P2X7 immunoreactivity. Scale bar, 50 µm.

5.5 Discussion

Neuropoietic cytokines are secreted by Schwann cells and macrophages, and elevated in DRG neurons upon peripheral nerve injury (Seth et al., 2009). Also, Schwann cells are a

rich source of neurotrophic factors such as nerve growth factor (NGF) (Henderson et al., 1993) and brain-derived neurotrophic factor (BNDF) (Koliatsos et al., 1993). Furthermore, ATP can be secreted by Schwann cells, through both anion transporters and calcium-dependent exocytosis, upon activation of purinergic receptors such as P2Y2 (Liu et al., 2005). It has also been reported that ATP released from nerves stimulates the ATP release from Schwann cells, and that they exchange positive feedback (Ellis, 2001). Therefore, we speculate that Schwann cells play an important role in the ATP-mediated conditioning-like effects. It is highly possible that when ATP is injected into the sciatic nerve, it stimulates purinergic receptors on Schwann cells and this can lead to increased release of neuropoietic cytokines/neurotophic factors and subsequent signalling for regeneration. We have consequently identified purinergic receptors on neurons and Schwann cells as the focus of this chapter.

Purinergic receptors are widely expressed in the nervous system. Ionotropic P2X receptors are known to regulate synaptic signalling (Larson and McPherson, 2001), pain (Rastinejad, 2001) and respiration (Schreyer and Skene, 1993). Indeed, P2X receptors were first described in sensory neurons (Nihalani et al., 2000) where they are valuable for neurotransmission and nociception (Fyffe and Perl, 1984). For P2X receptors, it has been reported that P2X1, P2X2, P2X3, P2X4, P2X5 and P2X6 receptors are present in DRG neurons in mRNA level using in-situ hybridization method (Kobayashi et al., 2005), and this is consistent with our realtime PCR data showing that all types of P2X receptors exist in DRG neurons.

P2X receptor expression in DRG was reported by Xiang et al. (1998) using antibodies produced by Roche. The levels of expression of P2X1-6 receptors identified by immunohistochemical methods were similar to our data. Also, the distinctive feature of

P2X3 receptor subtype expression - that it is much more intense in relatively small diameter neurons – has been observed by us and others (Xiang et al., 1998). These P2X3-expressing small neurons are believed to be thin and unmyelinated C-fibres conducting nociceptive signals (Xiang et al., 1998).

Others reported the size distributions of P2X receptors, other than P2X3 receptor subtype, on DRG neurons. For example, P2X2 mRNA is mainly expressed by small diameter neurons, while P2X5 and P2X6 mRNA preferentially are expressed by large size neurons (Kobayashi et al., 2005). However, our immunohistochemical data suggested that these receptors are evenly distributed in all sizes of neurons. Such discrepancies might be due to different experimental conditions. Further optimization of the immunohistochemistry such as using a different blocking solution might narrow the gaps between our data and others.

P2X7 mRNA was also detected in DRG neurons, but it was reported that P2X7-expressing DRG neurons were quite rare (Kobayashi et al., 2005), which corresponds with our data that P2X7 mRNA levels in DRG were the lowest among P2X receptors. However, P2X7 expression on DRG neurons in protein level is controversial: while Chen et al. reported that P2X7 is not expressed on DRG neurons but satellite cells (Wildman et al., 1999b), our immunohistochemical data suggested that P2X7 receptors were expressed at a just detectable level on DRG neurons. Our data also confirmed a strong expression of P2X7 receptors on satellite cells. In addition, in our data P2X3 and P2X4 receptors were expressed on satellite cells at a moderate level and P2X5 and P2X6 receptors were expressed at a just detectable level.

There is evidence for P2X receptors such as P2X1, P2X3 and P2X7 on Schwann cells from immunohistochemical studies in mice (Meyer et al., 1999, Colomar and Amedee, 2001). Our data also suggested that all types of P2X receptors are expressed in nerve fibres and Schwann cells of the sciatic nerve in various levels of immunoreactivity.

In regards to metabolic P2Y receptors, their roles in the nervous system are less well understood than P2X receptors. However, it is known that they modulate pain responses (Toole et al., 1999) and neuronal ion channels such as Ca^{2+} and K^{+} channels (Groschel-Stewart et al., 1999). In mRNA level, mRNA for P2Y1 and P2Y2 is expressed at a high level and P2Y4 and P2Y6 is expressed at a low level in rat DRG neurons in other studies (Newton et al., 1999, Townsend-Nicholson et al., 1999) and these results correspond with our realtime PCR data (P2Y2 > P2Y1 > P2Y14 > P2Y6 > P2Y13 > P2Y12 > P2Y4).

At protein level, our data showed the presence of all types of P2Y receptors in DRG neurons at various expression levels from very strong to just detectable. For P2Y1 receptors, although others have reported that P2Y1 receptors are expressed mostly in small size neurons which are neurofilament-negative (Wildman et al., 1999b, Ruan and Burnstock, 2003), our immunostaining data suggested that P2Y1 receptors are widely expressed at moderate level among neurons of all sizes. It was also reported that only a few satellite cells expressed P2Y1 receptors (Wildman et al., 1999b), but the P2Y1 immunoreactivity was absent in satellite cells in our data.

For P2Y4 receptors, the observation that a P2Y4 receptor antibody stained more medium and large size neurons (neurofilament-positive) than small size neurons (neurofilament-negative) (Ruan and Burnstock, 2003) was not reproduced by our

experiments. Our data suggested the universal P2Y4 expression on DRG neurons at a moderate level.

Studies using agonists suggested that at least the P2Y1, P2Y2 and P2Y4 receptors are present in satellite cells in mice (Wang et al., 2013). Our immunostaining data from rats showed P2Y2, P2Y4, P2Y6 and P2Y12 receptors expression in satellite cells.

Also, it was suggested that isolated Schwann cells from mammalian nerve trunks as well as Schwann cells in vivo in mammalian sensory endings possess P2Y2 receptors (Xiang et al., 1999, Wildman et al., 1999a, Lyons et al., 1994). Later, immunohistochemical studies confirmed the presence of the P2Y2 receptor subtype on rat Schwann cells (Liu et al., 2005). Studies using agoinsts also suggested the presence of P2Y1 and P2Y2 receptors on rat Schwann cells (Mayer et al., 1998). Our data showed the presence of all types of P2Y receptors in Schwann cells and nerve fibres of the sciatic nerve at various expression levels from very strong to just detectable.

P1 adenosine receptors, consisting of four subtypes, play an important role in synaptic modulation of neuronal activities (Kaelin-Lang et al., 1998). Expression of P1 receptors are not well characterised to date. Others have reported the presence of the A2a receptor subtype in large size DRG neurons in rats (Kaelin-Lang et al., 1998). Our immunohistochemical data suggested that A2a receptors were widespread in DRG neurons of all sizes at a moderate level. Our data showed all types of P1 receptors in DRG neurons. A1 receptor expression was the strongest among them. We have found A1, A2a and A2b receptors in satellite cells and their expression levels were similar.

Studies using analogues/antagonists suggested the presence of A2 receptors in the rat sciatic nerve (Sheldon et al., 1996). In our data, all four adenosine receptors were

identified in nerve fibres in the sciatic nerve and A2b and A3 receptor immunoreactivities were weaker than the A1 and A2a. All types of P1 receptors were expressed in Schwann cells, and all of them exhibited moderate levels of immunoreactivities, except A3 which showed just detectable immunoreactivity.

It is noteworthy that the most dominant purinergic subtype in the sciatic nerve is P2Y2, which is involved in neuronal development and regeneration (Arthur et al., 2005), whereas P2X4 is the major subtype in cultured Schwann cells. The substantial effects of cell culture on the expression of purinergic receptors and the complexity of cell types in sciatic nerve may explain the different pattern of purinergic receptors expression between the sciatic nerve and the Schwann cells.

There were some discrepancies between qPCR and immunohistochemistry data. It is thought to be due to the limitations of using immunohistochemistry for quantification of corresponding protein. Although immunohistochemistry is the best option for cellular localisation and morphology examination, western blot might provide more precise data for the amounts of each purinergic receptor in the tissue at protein level. Also, using specific software such as Matlab to quantify the intensities of immunoreactivity might be another option (Matkowskyj et al., 2000).

The critical aspect of the primary antibody is the specificity for the epitope of the targets. In this study, we have used non-primary antibody control (PBS was applied instead of primary antibody) and antigen pre-absorbing control to check the specificity of the primary antibodies. Also, the use of western blot to check whether only one specific band is seen when applying each antibody might provide more information. Using antibodies from different sources to evaluate whether they provide the same data could be another approach,

although there is only very limited availability of the antibodies for purinergic receptors. However, the best way to prove the specificity of the antibodies is to use the tissues from purinergic receptor knock-out animals. We could only obtain tissues from P2X7 receptor knock-out mice so far. Testing antibodies on more purinergic receptor knock-out mouse lines will provide the evidence for the specificity of the antibody and the assurance of the immunohistochemical data obtained in this study.

In the immunohistochemical data, we could observe some nuclear staining of several purinergic receptors such as P2Y1, P2Y12 and P2Y14. This could be non-specific binding, however, it could be the real staining of receptors with unknown functions. In fact, others reported P2X7 receptor nuclear staining in visceral smooth muscle cells in guinea pigs (Menzies et al., 2003).

Since most of the purinergic receptor subtypes were detected in the sciatic nerve, the next step would be to select the receptors to test for their agonists/antagonists prioritising them based on the level of expression and their reported relationship to regeneration. The P2Y2 receptor subtype is particularly interesting since it is known to be involved in neuronal development and regeneration (Arthur et al., 2005). P2Y2 transcripts are most dominant among all purinergic receptors in the sciatic nerve and P2Y2 receptors are strongly expressed in protein levels in both Schwann cells and nerve fibres of the sciatic nerve. It would be interesting to assess the effects of agonists/antagonists of P2Y2 receptors on neurite outgrowth in vitro and axonal regeneration in vivo.

CHAPTER 6 - GENERAL DISCUSSION

6.1 Summary of experimental findings

The overall aim of this thesis was to investigate whether intraneural injection of ATP into the sciatic nerve can mimic the effects of a conditioning lesion by enhancing growth capacity of sensory neurons. The summary of the key findings regarding intraneural injection of ATP is as follows.

- Injection of ATP into the adult rat sciatic nerves significantly promoted neurite outgrowth of dissociated DRG neurons and induced a significant increase in the number of ascending sensory axons growing into the lesion cavity compared with a saline injection group in a dorsal column transection model, indicating ATP injection could mimic the effects of conditioning lesion.
- CNTF and IL-6 levels were increased at the injection site and DRG after intraneural ATP injection. Also, ATP injection activated axon growth related transcription factor STAT3 and increased the expression of GAP43 in DRG neurons, indicating JAK/STAT3 pathway is involved in this ATP mediated growth promoting effects.
- A second ATP injection (ATP/ATP) one week later triggered a 166-fold increase in the number of sensory axons growing into the lesion centre compared with the double saline injection group (saline/saline). Some of the regenerating axons of ATP/ATP group extended into the rostral spinal cord as far as 1.75 mm, while no axon in the ATP/saline and saline/saline groups grew into the rostral spinal cord.
- Sustained STAT3 activation and GAP43 expression for as long as three weeks were observed after double ATP injections.
- Intraneural ATP injection showed mild Wallerian degeneration at the injection site, however, no long-term adverse effects on the motor and sensory functions of the sciatic nerve were observed.

- Purinergic receptors in the sciatic nerve were identified in order to find the receptor(s) responsible for the ATP mediated conditioning-like effects. High levels of mRNA transcripts of P2X4, P2Y2 and P2Y13 were detected in cultured Schwann cells. Transcripts of P2Y2, P2Y13, P2Y14, A1 and A2a were predominant in the sciatic nerve and transcripts of P2X3, P2Y2, A1 and A2a were predominant in DRG. In protein level, DRG neurons showed strong immunoreactivity of P2X1, P2X3, P2X4, P2X6 and A1 receptors, while satellite cells showed strong immunoreactivity of P2X7 and P2Y2 receptors. Axons in the sciatic nerve showed strong immunoreactivity of P2Y2 receptors, while Schwann cells showed strong immunoreactivity of P2X7, P2Y2, P2Y12 and P2Y13 receptors.

Our data suggest that intraneural ATP injection, especially double injections, can be a potent potential therapeutic approach in treating patients with spinal cord injury.

6.2 Limitations of the study and other options to overcome the limitations

In this study, we used a dorsal column transection as an in vivo model. Dorsal column transection is a helpful model to understand the underlying mechanisms of central axonal regeneration and identifying experimental effects of any therapeutic approaches for promoting axonal regeneration (Tuszynski and Steward, 2012). Even though functional deficits can be assessed after injury, axonal regeneration has to reach all the way to the targets in the medulla oblongata (nucleus gracillis) to restore sensory function (Tuszynski and Steward, 2012).

As we only used one in vivo model it would be valuable to check whether ATP injection is also effective in other types of spinal cord injury such as contusion and

compression injury which are clinically more frequent. Furthermore, we only tested the effects of ATP injection on thoracic spinal cord injury. It would be interesting to examine the effects on spinal cord injury at other levels such as the cervical injury. In fact, a high cervical injury model can be useful for the evaluation of sensory functional recovery as the distance between injury and the functional target is much shorter. Others have reported sensory axonal regeneration back to the dorsal column nuclei with high cervical injury models (Alto et al., 2009, Bonner et al., 2011).

Following the ATP injection in our experiments, even in double injection groups, axons grew into the rostral spinal cord beyond the lesion cavity but only as far as 1.75 mm which is not enough to achieve successful functional regeneration. To achieve functional recovery, we may need to try combined therapy with other treatments, such as making CNS environments more permissive using PSA or myelin inhibitor antibodies, using cell transplantation or delivering neutrophins. Also, using more potent P2 agonists than ATP or using ecto-ATPase inhibitors in company with ATP could be the other methods to achieve more effective axonal regeneration.

In addition, we did spinal cord injury and ATP injection at the same time, which will not happen clinically. Although it is possible for limited numbers of patients to receive regenerative interventions soon after injury, many patients are either too ill or it is difficult to fit in other treatments into the clinical plan in the acute period of the spinal cord injury. For clinical use, optimal time point for the regenerative treatment would be in the subacute phase (typically 2-6 weeks after injury) (Wang et al., 2011). Therefore, what we need to do next is to test the effects of ATP injection in subacute and chronic conditions of spinal cord injury.

Our results only show that ATP can stimulate the DRG neurons. However, cell bodies of DRG neurons are in periphery. To test whether the ATP injection also has an effect on real CNS neurons would be interesting. Moreover, as conditioning effects are known to hold true not only for sensory axons but also for motor axons (McQuarrie, 1978, Jacob and Croes, 1998, McQuarrie, 1986), it would also be interesting to see whether ATP injection can act on motor neurons.

6.3 Unanswered questions and future directions

The main aims of this thesis were to understand the underlying the mechanism of the conditioning lesion and to test the hypothesis that ATP is the key mediator of the conditioning lesion. Although intraneural injections of ATP mimicked the effects of the conditioning lesion, upregulating various molecules which are closely related to the neuroregeneration, there are still questions unanswered and the results obtained raised interesting new questions.

ATP injection upregulated neuropoietic cytokines CNTF and IL-6 in the DRG and sciatic nerve (injection site), mimicking a conditioning lesion (sciatic nerve crush). However, the patterns of the upregulation of two cytokines were slightly different, even though they signal through the same JAK/STAT3 pathway. While CNTF levels in the ATP injection group were higher than that in the crush group in both DRG and sciatic nerves, IL-6 levels in the ATP injection group were either similar to the level of the crush group in DRG or lower than that of the crush group in sciatic nerves.

One possible reason would be that different types of cells are associated in the synthesis and release of these cytokines. The main source of CNTF is thought to be

Schwann cells as axotomy induced the increase in the synthesis and release of CNTF in Schwann cells (Sendtner et al., 1992). IL-6 can be released by recruited macrophages (Rotshenker, 2011) as well as neurons (Murphy et al., 1995). ATP triggered release of IL-6 in fibroblasts was also reported (Solini et al., 1999). Which cell types are responsible for the upregulation of each cytokine is still unclear. However, there is a point to make that earlier response has to be made largely by resident cells as it takes 2-3 days for macrophages to migrate to the nerve injury site and peak time of the migration is 1 week after injury (Rotshenker, 2011).

Moreover, peak time points of release or synthesis of the two cytokines might be different. Measuring the levels of these cytokines in various time points will provide more information of their dynamic changes after the treatments. Also, the technique we used (ELISA) measures the levels of the cytokines from both inside and outside of the cells. Using other techniques such as immunohistochemistry might determine the cellular localization of the cytokines. In addition, the reason for relatively small increase of CNTF after sciatic nerve crush could be the disruption of the axonal transport of CNTF due to the nerve damage.

LIF, another cytokine involved in the JAK/STAT3 pathway, is also strongly implicated in the conditioning lesion (Cafferty et al., 2001) and it can be synthesised and released by either Schwann cells or fibroblasts (Subang and Richardson, 2001). Also, it was reported that ATP induced LIF mRNA in astrocytes (Yamakuni et al., 2002). It might be interesting to check the changes of LIF after ATP injections in different time points.

ATP did not change the levels of ATF3 or pcJun 3 days after injection. However, it is possible that we have missed the peak time points for upregulation of these transcription

factors after ATP injection. ATF signals can be detected 12 hours after axotomy, and the signals increase to peak levels 1 day after axotomy and remain at this level upto 2 weeks (Tsujino et al., 2000). cJun increases can also be observed as early as 3-6 hours after axotomy and last over 21 days (Herdegen et al., 1998). Nevertheless, time points for peak levels of these transcription factors after ATP injection and axotomy could be different. Measuring the transcription factors at different time points could show us broader pictures of their changes after ATP injections. It would also be interesting to check how long pSTAT3 and GAP43 upregulation can sustain after ATP injection by observing them for even longer period time than three weeks.

In addition, the examination for changes of these transcription factors and growth associated protein at the injection site will provide clearer views of how the molecules/pathways work in the sciatic nerve and DRG after ATP injection. For example, increased pSTAT3 at the injection site could explain that upregulation of neuropoietic cytokines leads to the activation of JAK/STAT3 pathway at the injection site and these molecules are retrogradely transported to the neuronal cell bodies in DRG (Shin et al., 2012), which then activates the transcription of the pro-regenerative genes.

We have not measured the changes of pSTAT3 and GAP43 after ATP injection in other subpopulations of DRG neurons such as $CGRP^{+}$ neurons (Lawson, 1992). It would be valuable to understand whether ATP injection affects other subpopulations of DRG neurons.

The concentration of ATP (150 μM) was arbitrarily chosen. All other P2 receptors, except P2X7, can be activated at relatively lower concentration of ATP. The main goal of using 150 μM of ATP was to prevent P2X7 receptors from opening the pores that allow the passage of molecules with larger molecular weight, which is thought to be involved in the

induction of apoptosis (Burnstock, 2007a). However, optimal concentration of ATP for injection is not determined. It might be critical to find that to promote regeneration more effectively.

Further approaches to uncover the underlying mechanisms of ATP would be really intriguing. We only checked limited numbers of molecules/pathways after ATP injection in this thesis. However, more molecules/pathways can be involved in ATP-mediated growth promoting effects. For example, we may check cAMP/PKA/CREB signalling, which is involved in the effects of a conditioning lesion (Cai et al., 2001, Cai et al., 2002, Lu et al., 2004, Neumann et al., 2002, Gao et al., 2004), after ATP injection. In the thesis, we have focused on the changes in DRG neurons after ATP injection. However, ATP injection (or conditioning lesion) might change some molecules/pathways in neurons in the CNS. In that case, the application of ATP injections would be much more extensive.

Whether injections of ATP into the tibial nerves or peroneal nerves elicit significant regenerative responses would be an interesting question. Then, how about injecting into the even smaller branches of the nerves? It might significantly reduce the side effects of nerve damage after injection. Electrical stimuli in the skeletal muscle are known to release ATP (Osorio-Fuentealba et al., 2013) and, furthermore, gentle stimulation of the nerve (such as shear stress) can release ATP (Burnstock, 1999). Therefore, alternative approaches to increase ATP levels in the extracellular space without causing any damage may be found in the future.

In Chapter 5, profiles of purinergic receptor subtypes in non-injured animals were investigated. However, these profiles can be changed after central or peripheral nerve injury as well as ATP injection. It might be very interesting to characterise the upregulation or

downregulation of the purinergic receptor subtypes after an injury or ATP injection. Western blotting technique might be a good option for the comparison of the upregulation or downregulation of each receptor after interventions. Dramatic changes of certain receptor subtypes after ATP injections might provide useful answers for an important unanswered question of which specific receptor subtype(s) are involved in ATP-mediated conditioning-like effects.

Using specific agonists and antagonists for each purinergic receptor present in the sciatic nerve could also provide the answers. We have set up a compartmented co-culture system using Campenot chambers. This in vitro system separates the environment of the cell body and the neurites of the DRG neurons, and allows us to mimic the CNS (co-culturing with astrocytes or dissociated spinal cord cells, can be pre-coated with myelin inhibitors) in one outer compartment and the PNS (co-culturing with Schwann cells) in another outer compartment (Figure 6-1 a). In our data, neurites of DRG neurons grew along the tracks under the Campenot chamber (Figure 6-1 b), allowing for the measurement of neurite outgrowth in the 'CNS' compartment after the treatment of agonists/antagonists of purinergic receptors applied in the 'PNS' compartment. Limitations would be that dissociated DRG and Schwann cells might act differently compared with an 'in vivo' situation and expression of purinergic receptors might be different. Also, neuropoietic cytokines can be diluted in culture medium in contrast to in vivo conditions, where cytokines from Schwann cells act directly on axons. However, these in vitro experiments will provide general information about the effects of agonists/antagonists of purinergic receptors.

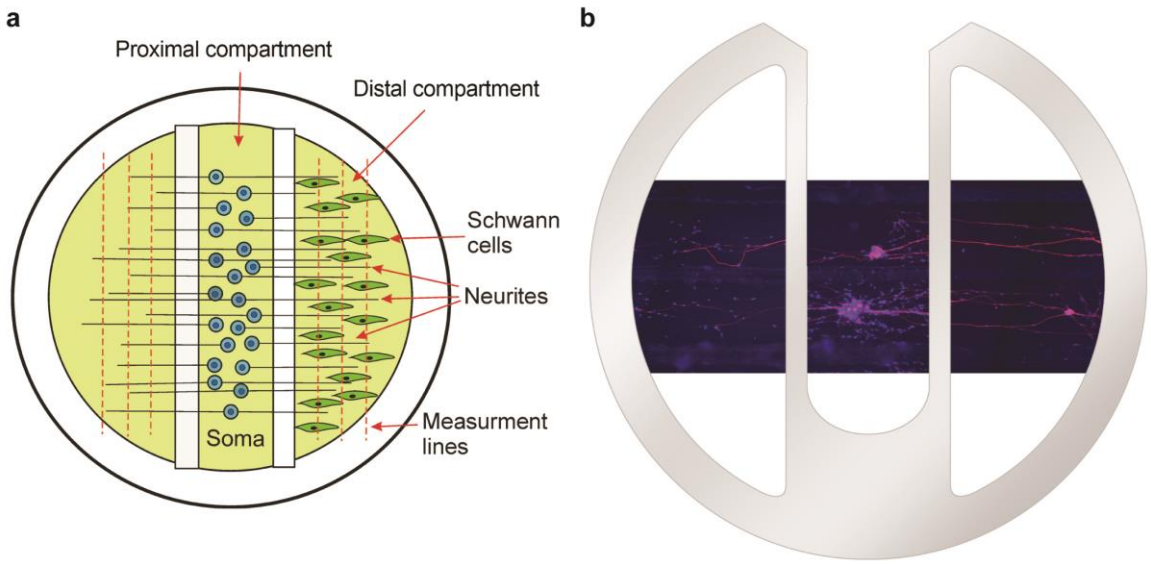

Figure 6-1 A compartmented DRG culture system using Campenot chambers. **a**, Schematic drawing for the in vitro test for the effects of agonists and antagonists of purinergic receptors on DRG neurons. **b**, DRG neurons grow along the tracks under the Campenot chamber. Red, immunostained with a mouse monoclonal antibody against βIII tubulin. Blue, counterstained by DAPI.

Our intuition is that more than one receptor would be responsible for the ATP-mediated conditioning-like effects. For example, P2X and P2Y receptors may cooperate to exert the actions as P2X receptors are fast-acting ionotropic receptors and P2Y receptors work through G proteins to various second messenger pathways mediating slow metabotropic actions (Ruan et al., 2005).

Based on the results from this in vitro experiment, our next step would be using available selective purinergic receptor knockout mice (ex. P2X7 knockout mice) to check whether the ATP-mediated conditioning-like effect diminishes or disappears in these

knockout mice. P2Y2 knockout mice would be the first ones to test the effects of ATP injection as P2Y2 receptors are highly expressed in the sciatic nerves at both mRNA and protein levels and strongly related to regeneration: P2Y2 activation leads to the GAP43 upregulation and activation of nerve growth factor/TrkA signalling (Arthur et al., 2005).

Completion of this work will contribute to the development of more potent therapeutic agents (agonists) to effectively stimulate the purinergic receptor(s) which are responsible for ATP-mediated growth-promoting effects. I anticipate that these drugs will someday be clinically administered for patients suffering from spinal cord injury.

I would like to close the thesis with a famous statement of Ramon y Cajal (1928): "In adult centers the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated" (Love, 2003). Thanks to the efforts of numerous scientists in the past, this is already considered largely untrue. It is the role of the next generation of scientists like us to make this statement truly false in the near future.

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